Hepatocellular injury, inflammatory response & metabolic changes after laparoscopic bariatric surgery

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Hepatocellular injury, inflammatory response & metabolic changes after laparoscopic bariatric surgery

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Abstract

Over 80% of patients undergoing bariatric surgery have non-alcoholic fatty liver disease (NAFLD), including a third with steatohepatitis. Fatty livers are vulnerable to toxic insults, including ischaemia-reperfusion injury (IRI) and inflammation. The induction of pneumoperitoneum and retraction of the liver during laparoscopic surgery causes an acute inflammatory response and IRI.

In this thesis, twenty patients were recruited to a randomised controlled trial of N-acetylcysteine (NAC), an antioxidant, given before and during laparoscopic sleeve gastrectomy (LSG) to reduce liver injury and post-operative inflammatory response. The acute effects of LSG were investigated, with measurement of cytokines, markers of hepatocyte death and oxidative stress.

The results showed that NAC did not significantly reduce the rise in liver enzymes or improve clinical outcomes. Further assays demonstrated that a significant inflammatory and oxidative stress insult occurs, with increases in markers such as C-reactive protein, IL-6, superoxide dismutase and cytokeratin-18 (CK-18) fragments, indicative of hepatocyte death. These markers returned to baseline within 48 hours. The patients were followed for 6 months and the result of longitudinal changes are described, showing significant reduction in BMI, along with reductions in insulin resistance, inflammatory markers and CK-18. Bile acid (BA) metabolism was also studied, showing a significant increase in fibroblast growth factor 19 levels, indicating a likely increase in bile delivery to the terminal ileum after LSG, which was associated with significant changes in four conjugated BA, although total BA was unchanged. These changes correlated with reduction in inflammatory markers and hepatocyte death.

In conclusion, LSG is associated with a short-term inflammatory response and liver injury. After 6 months, the relevant markers fall below baseline, indicating improvement in inflammatory profile, glycaemic control and extent of NAFLD. Further work is needed to understand these relationships in a larger sample of patients undergoing a variety of bariatric operations.

Declaration of Originality

The candidate and the supervisors obtained ethical approval for this study.

Ms Kirstin A Carswell, Clinical Research Fellow, and Mr Ameet Patel, Consultant Surgeon and First Supervisor, helped the candidate in the recruitment of participants, sample collection and clinical care of the patients.

Dr Royce Vincent performed the bile acid analysis and fibroblast-growth factor 19 ELISA.

The clinical blood tests, such as full blood count, liver function, insulin and lipid profiles, were performed in the King's College Hospital Departments of Haematology and Biochemistry on automated analysers as part of routine clinical care.

All of the analytical work (except for the measurement of plasma bile acids and FGF-19) and the statistical analyses in this thesis were carried out by the candidate.

'I, Ajay Prakash Belgaumkar, declare that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis."

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List of Abbreviations

5-PL	5 parameter logistic (type of curve fitting algorithm)
AA	Apoptosis Array
ACE	Angiotensin converting enzyme
AGB	Adjustable Gastric Banding
AHA	American Heart Association
ALT	Alanine aminotransferase
AMP	(cylic-) Adenosine monophosphate
ANOVA	Analysis of variance
ASMBS	American Society for Metabolic and Bariatric Surgery
AST	Aspartate transaminase
ATP	Adenosine triphosphate
AUC	Area under curve (calculated using trapezoidal rule)
AUROC	Area under the Receiver operator characteristic (curve)
BA	Bile Acid(s)
BMI	Body Mass Index (=weight in kg/(height in m)^2)
BPD	Biliopancreatic diversion
BSA	Bovine serum albumin
BT	Bile transport protein
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CI	Confidence Intervals (95%)
CK-18	Cytokeratin-18 fragment
CPAP	Continuous positive airway pressure
CRP	C-reactive protein
CT	Computed tomography
CTIMP	Controlled trial of investigational medicinal product
DCA	Deoxycholic acid
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DNP	Dinitrophenylhydrazine
DS	Duodenal switch
EBWL	Excess body weight loss
EDTA	Ethylenediaminetetraacetic acid
EIA	Type of buffer
ELF	Enhanced Liver Fibrosis panel
ELISA	Enzyme linked immunosorbent assay
EMEM	Eagle's minimal essential medium
EWL	Excess weight loss
FA	Fatty acids (usually Free Fatty Acids)
FADD	Fas associated Death Domain
FBG	Fasting blood glucose
FC	Fold Change
FFA	Free fatty acid (also known as NEFA)
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor

FXR	Farnesoid X receptor
GB	Gastric Bypass (various forms)
GCA	Glycocholic acid
GCBA	Glycine conjugated bile acid(s)
GCDC	Glycochenodeoxycholic acid
GDCA	Glycodeoxycholic acid
GGT	Gamma glutamyl transferase
GIP	Glucose-dependent insulinotropic peptide
GLCA	Glycolithocholic acid
GLP	Glucagon-like peptide (-1)
GPX	Glutathione peroxidase (enzyme)
GSH	Glutathione
GSSH	Reduced glutathione
GUDCA	Glycoursodeoxycholic acid
HA	Hyaluronic acid
HABP	Hyaluronic acid binding protein
$HBA1_c$	Glycated Haemoglobin (measure of glycaemic control)
HDL	High density lipoprotein
HIF-1	Hypoxia inducible factor 1
HO-1	Haemoxygenase 1
HOMA	Homeostasis Model Assessment (HOMA-IR =Fasting insulin x Fasting Glucose/22.5)
HPB	Hepato-pancreatico-biliary
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxidase
HS	High sensitivity
HSC	Hepatic stellate cells
HSP	Heat shock protein
IAP	Inhibitor of apoptosis
ICG	Indocyanine green
IDF	International Diabetes Federation
IDN-6556	Name of a pan-caspase inhibitor
IFG	Impaired fasting glucose
IFN	Interferon
IL	Interleukin
IMP	Investigational medicinal product
INR	International normalised ratio (of clotting)
INT	Iodonitrotetrazolium
IPC	Intermittent preconditioning
IQR	Interquartile range
IR	Insulin Resistance
IRI	Ischaemia-reperfusion injury
IU	International units
JCTO	Joint Clinical Trials Office (monitoring of trial)
LAGB	Laparoscopic Adjustable Gastric Banding
LC	Liquid chromatography
LCA	Lithocholic acid
LCPUFA	Liver n-3 long-chain polyunsaturated fatty acid
LDL	Low density lipoprotein

LOD	Limit of detection
LOS	Length of (hospital) stay
LPS	Lipopolysaccharides
LRYGB	Laparoscopic Roux-en-Y gastric bypass
LSG	Laparoscopic sleeve gastrectomy
LXR	Liver orphan receptor
M30	Cytokeratin-18 fragment M30 epitope
M65	Cytokeratin-18 fragment M65 epitope
MDA	Malondialdehyde (marker of lipid peroxidation)
MHRA	Medicines and Healthcare products Regulatory Agency
MR	Magnetic resonance
MRI	Magnetic resonance imaging
MRM	Multiple reactions monitoring
MS	Mass spectrometry
NAC	N-acetylcysteine
NAC Trial	Randomised Trial of NAC in laparoscopic bariatric surgery
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD Activity Score (by Brunt and Kleiner)
NASH	Non-alcoholic steatohepatitis
ND	Not described
NEFA	Non-esterified fatty acids (also known as FFA)
NF	Nuclear factor
NHLBI	National Institute of Health Heart Lung and Blood Institute
NICE	National Institute of Clinical Excellence
NK	Natural killer T cells
NKT	Natural killer T cell
NO	Nitric oxide
NPV	Negative predictive value
NPY	Neuropeptide Y
OBCA	Obeticholic acid
OBS	Observational Studies
OH	Hydroxyl moeity
OR	Odds ratio
OS	Oxidative stress
OSA	Obstructive sleep apnoea
OXM	Oxyntomodulin
PBS	Phosphate buffered saline
PC	Protein carbonyl
PE	Phycoerythrin
PLT	Platelets
PON-2	Protein paraoxonase 2
PP	pneumoperitoneum
PPV	Positive predictive value
PVAT	Perivascular adipose tissue
PXR	Pregnane X receptor
PYY	Peptide YY
RCT	Randomised controlled trial

RNA	Ribonucleic acid
ROC	Receiver operator characteristic
ROS	Reactive oxygen species (also known as free radicals)
RPM	Revolutions per minute
RXR	Retinoid X receptor
RYGB	Roux-en-Y Gastric Bypass (laparoscopic or open)
SD	Standard deviation
SDS	Sodium dodecyl 10 sulphate
SEM	Standard error of the mean
SG	Sleeve gastrectomy
SHP	Small heterodimer protein
SOD	Superoxide dismutase
SPSS	Statistical Package for Social Sciences
SS	Simple steatosis
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Taurocholic acid
TCBA	Taurine conjugated bile acid(s)
TDCA	Taurodeoxycholic acid
TE	Transient elastography
TG	Triglycerides
TLCA	Taurolithocholic acid
TLR	Toll like receptor pathway
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TRADD	TNF-related
TRAIL	Tumour Necrosis Factor related apoptosis inducing ligand
TRAP	Total radical antioxidant parameter
TUDCA	Tauroursodeoxycholic acid
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TWEAK	TNF receptor type 1-associated death domain
UCP	Uncoupling protein
UDCA	Ursodeoxycholic acid
UK	United Kingdom
US	United States (of America)
USA	United States of America
VBG	Vertical banded gastroplasty
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
WCC	White blood cell count
XIAP	X-linked inhibitor of apoptosis
γGT	Gamma glutamyl transferase

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This thesis is dedicated to my family, who have patiently supported me during the course of my research post and following my return to clinical work. Thanks especially to my wife, Roshi, who has had to work harder than anyone else to enable me to complete this thesis. My wonderful daughters, Leila and Anjali, were born after the inception of this research work and I hope they will be able to enjoy the fruits of this labour.

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Finally, thanks to my senior research colleagues and supervisors for their guidance, support (and funding!): Mr Ameet Patel, Professor Anil Dhawan, Dr Robin Hughes and Dr Ragai Mitry.

Hypothesis & Aims

The pathophysiology of obesity links inflammation and oxidative stress with insulin resistance and fatty liver disease. Bariatric surgery leads to significant weight loss. The surgery itself provokes an inflammatory response. Fatty livers are more vulnerable to toxic insults, especially factors that might cause ischaemia-reperfusion injury. The inflammatory response to laparoscopic bariatric surgery, in the context of fatty liver disease, has not been specifically investigated previously.

The main hypothesis at the outset of this work is:

Administration of N-acetylcysteine will reduce the post-operative inflammatory response and liver injury after laparoscopic bariatric surgery.

This thesis sets out to answer the following questions:

- Does bariatric surgery have a significant impact on markers of oxidative stress, inflammation and liver injury in morbidly obese patients?
- What is the relationship between liver injury and oxidative stress and inflammatory markers?
- Do these relationships change in the short and medium-term (6 months) after surgery?

AIMS OF THIS THESIS

1. To review and summarise the available literature, including relevant background information, on the effect of bariatric surgery on obesity and fatty liver disease, in the context of inflammatory response, chronic inflammation and oxidative stress
2. To quantify the changes in biomarkers of oxidative stress, inflammation and liver injury both acutely and over a longer time period after bariatric surgery
3. To investigate the use of an antioxidant, N-acetylcysteine, to ameliorate the immediate post-operative response
4. To determine the relationships between these markers and pre- and intra-operative factors, and on post-operative outcomes
5. To explore the effect of surgery on fatty liver disease and identify relationships with other metabolic changes, including insulin resistance and bile metabolism

Overview of Thesis

Chapter 1 – Bariatric Surgery, Obesity and Inflammation

A general introduction of bariatric surgery is given, including a brief historical recap, along with an overview of the metabolic impact of surgery. There is a particular emphasis on the pathophysiology of obesity related inflammation and insulin resistance.

Chapter 2 – Fatty Liver Disease

Relevant background information is given about the pathology of NAFLD, the particular vulnerability of fatty liver to ischaemia-reperfusion injury and the effect of bariatric surgery on progression from NAFLD to NASH.

Chapter 3 – Liver Injury and Pneumoperitoneum

The introductory chapters are concluded with a review of the literature on laparoscopy-induced injury to the liver and the association with oxidative stress and inflammatory response. These introductory chapters give a comprehensive background to the relationship between obesity, fatty liver, inflammation and the response to laparoscopic bariatric surgery.

Chapter 4 – The effect of intraoperative N-acetylcysteine on hepatocellular injury during laparoscopic bariatric surgery. A randomised controlled trial.

This trial forms the main original work of this thesis. The chapter includes the trial methodology, the clinical data and primary outcomes of the trial.

Chapter 5 – Acute changes in oxidative stress, cytokines and hepatocyte death after laparoscopic bariatric surgery.

This chapter includes an overview of the biomarkers used to assess the acute pathophysiological changes after surgery in the NAC Trial.

Chapter 6 – The effect of laparoscopic sleeve gastrectomy on bile acids, markers of inflammation, oxidative stress and liver injury after 6 months.

A review of the existing literature pertaining to bile acids (BA) changes after bariatric surgery is given. The results describe the changes in metabolic profiles and circulating biomarkers after LSG and the effect on BA, and their relationship to markers of liver injury and inflammation.

Chapter 7– Conclusions.

The thesis is concluded with a discussion of the main results of each group of experiments and attempts to amalgamate those results together. The future direction of this work is described, with suggestions for improvement in study design.

CHAPTER 1

BARIATRIC SURGERY, OBESITY AND INFLAMMATION

1.1 Aims of Chapter

The aim of this chapter is to describe the background and evolution of bariatric surgery and set this in the context of modern surgical practice. The chapter goes on to explain the link between obesity, insulin resistance and chronic low-grade inflammation, with a review of the literature on the beneficial effects of bariatric surgery.

1.1.1 Bariatric Surgery - A definition

In a historical sense, obesity was not perceived or characterised as a disease **(1)**. The vast majority of the population in pre-industrialised societies were malnourished. Being overweight was a sign of wealth and affluence. Following the move away from an agrarian lifestyle, obesity began to be recognised as a cosmetic issue in the late 19th century **(2)**. Today, high-calorie, highly processed food is easily available in Europe and North America. Lifestyles are increasingly sedentary. Along with cultural influences, these factors have lead to an increasingly high prevalence of obesity in the general population **(3)**. The impact of increasing levels of obesity includes higher rates of chronic diseases, including diabetes mellitus, hypertension, cardiovascular disorders and osteoarthritis. Obesity is now described as a disease – an entity with symptoms that requires specific treatment **(4)**. For a majority of the obese population, control of weight through behavioural modification is unsuccessful **(5)**. Over the last 70 years, surgical and pharmacological interventions have developed to combat weight gain and effect weight loss, in an effort to reverse or attenuate the effects of obesity **(6)**. The term "bariatric" is derived from the Greek "baros" meaning "weight". Bariatric surgery refers to the subspecialty branch of gastrointestinal surgery concerned with causing deliberate weight loss in an effort to improve the patient's health.

1.2 A brief history of Bariatric Surgery

Bariatric surgery was first popularised in the United States of America **(7)**. The precise reasons are due to cultural, historical and financial factors that are outwith the scope of this thesis, and this is a brief synopsis. By the 1950s, increasing numbers of patients were reported to be surviving extensive emergency small bowel resections, with varying levels of post-operative morbidity **(8)**. The rising incidence of obesity in the USA led to increasing pressure on medical services to treat this new disease **(9)**. In a fee-for-service system, medical practitioners were economically incentivised to innovate treatments for this group of patients **(10)**.

1.2.1 Experimenting with jejuno-ileal bypass

Arnold Kremen and colleagues at the University of Minnosota devised a series of experiments on dogs in the early 1950s to define more clearly the relative importance of different sections of the small bowel in maintaining health **(11)**. Ten groups of dogs were subjected to different variations of the surgery, which involved taking 50-70% of the small bowel out of circuit (by exterioring the ends to the skin) and anastomosing proximal small bowel to the distal end, including variations that preserved or bypassed the ileo-caecal valve. Some dogs underwent two operations. Weight, intestinal transit times and faecal fat content were measured serially. Kremen *et al* concluded that up to 70% of the small bowel could be removed, that sacrifice of the ileum lead to significant fat malabsorption and preservation of the ileo-caecal valve lengthened transit times. Most importantly, they demonstrated that jejuno-ileal bypass was relatively safe and the dogs, in general, remained alive and reasonably healthy. In their conclusion, the authors mentioned that they had already begun to apply these findings to patients **(11)**. A letter published in reply to the study enthusiastically stated "…this questionable method of controlling obesity will have the necessary experimental foundation" **(11)**. Notably, long-term data beyond 24 weeks were not presented and the dogs did not have detailed evaluation of biochemical and haematological parameters.

Over the next twenty years, various configurations of jejuno-ileal bypass were performed **(12)**. These malabsorptive procedures were associated with dramatic

weight loss but also dangerous nutritional deficiencies and a significant risk of liver cirrhosis and failure. Many patients required reversal of the bypass, leading to weight regain. A second strain of weight loss procedures developed, involving restriction of intake by modification of the stomach and/or gastro-oesophageal junction **(13)**. Buchwald has written a comprehensive overview of the development of these different modes of weight loss surgery **(6)**. He uses the terms of evolutionary biology to liken developments in malabsorptive and restrictive surgeries to different genealogies.

There was a plethora of small anatomical variations in jejuno-ileal bypass operations, all of which cause weight loss by reducing the absorptive capacity of the small bowel **(6)**. It is striking that most of these operations were devised on a virtually experimental basis and performed in patients without any clear, objective prior evidence of their safety and efficacy. These short case series are as much advertisements for a particular technique as scientific reports and all of the described procedures have long been abandoned because they proved to be dangerous or ineffective. In the 1970s, jejuno-ileal bypass was widely performed. Ninety percent of patients achieved significant weight loss, but complications rates were high **(13)**. Most patients had troublesome side effects, including intractable diarrhoea and sequelae of malnutrition, leading to a high proportion of patients undergoing reversal surgery **(14)**.

1.2.2 Introduction of gastric bypass for weight loss

Mason and Ito at the University of Iowa began to perform gastric bypass (GB) in the late 1960s **(15)**. Subtotal Gastrectomy with gastrojejunostomy was increasingly used to treat duodenal ulcers. These patients experienced significant restriction of intake, troublesome post-prandial symptoms of "dumping" and relief of their ulcer symptoms, all of which led to weight loss. A series of dog experiments in a histamine-induced ulcer model confirmed their findings that GB leads to thickening of the antral mucosa and prevents further ulceration in both duodenum and jejunum **(16)**. The initial report on 34 patients concludes that GB leads to significant weight loss although they did not recommend it for treatment of ulcers alone **(15)**. Subsequently, GB was also used to treat intractable peptic

ulceration when other types of non-resectional anti-reflux surgery had failed. GB was then modified by Alden to include a Roux-en-Y reconstruction. In 1977, three centres reported comparative studies of GB versus jejuno-ileal bypass that showed GB was safer, with less frequent and less serious complications **(14, 17, 18)**. Griffen *et al* also noted that the incidence of liver disease was lower in their GB group **(14)**.

Scopinaro reported results of biliopancreatic diversion (BPD) in 1979 **(19)**. This included a partial Gastrectomy with Roux-en-Y reconstruction and preserved flow (either enteric or bilio-pancreatic) through all segments of bowel. Weight loss and resolution of co-morbidities was very impressive although there was still a significant incidence of protein malnutrition. BPD was subsequently adapted by Marceau in Canada to include a sleeve Gastrectomy and duodenojejunostomy – a duodenal switch (DS) **(13)**. By preserving the pylorus, transit of food into the ileum was slowed, reducing diarrhoea and cramping.

Restrictive surgery also developed over the same time period **(7)**. Mason introduced partial partition of the proximal stomach, stapled gastroplasty, which he felt was safer and quicker to perform. By the 1980s, the most widely accepted restrictive procedure was the vertical banded gastroplasty (VBG) **(12)**. VBG had a good safety profile and resulted in significant early weight loss. However long-term weight regain was common. The principle of proximal restriction just below the gastro-oesophageal junction has evolved to include gastric banding operations **(6)**. The development of high grade plastics facilitated the design of the adjustable gastric band, which is an inflatable synthetic band that can be wrapped around the top of the stomach, restricting the aperture just below the gastro-oesophageal junction **(19, 20)**. These operations are safe, the bands are removable and weight loss results are good although less dramatic than GB or BPD-DS.

1.3 Modern Bariatric Surgery

Over the last 10 years, the numbers of patients undergoing bariatric surgery in the United Kingdom has grown exponentially **(21)**. As evidence about the safety, tolerability and efficacy of the operations has mounted, clinicians and patients

have sought surgical treatment with enthusiasm **(22)**. In contrast to the United States, where open bariatric surgery had already been popularised by the 1980s **(23)**, the introduction of minimally invasive or laparoscopic techniques was crucial to its broadening acceptance in the UK **(21)**.

Laparoscopic gastric bypass surgery is associated with shorter length of stay, lower rates of complications and mortality and lower hospital costs **(24, 25)**. Respiratory complications are lower after laparoscopic rather than open surgery (0.94% versus 3.87%, respectively; $p<0.01$) **(26)**. Laparoscopic bypass surgery is also associated with a decreased stress response, with lower post-operative C-reactive protein (CRP) and Interleukin (IL-) 6 levels, despite longer operating times **(27)**.

Starting with the US National Institute of Health Consensus Statement regarding the safety and efficacy of bariatric surgery in 1991, an attempt has been made to standardise and rationalise the various types of surgical procedures **(28)**. By 2004, the American Society for Metabolic and Bariatric Surgery (ASMBS) had also issued a consensus statement agreeing that four operations were deemed acceptable and established treatments: Roux-en-Y gastric bypass (RYGB), laparoscopic adjustable gastric banding (LAGB), vertical banded gastroplasty (VBG) and biliopancreatic diversion and duodenal switch (BPD/DS) **(29)**. In the UK, the National Institute of Clinical Excellence also issued guidance, recommending surgery in suitable and motivated patients who had failed to lose weight by non-surgical interventions **(30)**.

Surgery can be offered to patients with a BMI over 40 kg/m^2 or in patients with a BMI over 35 kg/m^2 who have associated complications, such as diabetes, hypertension, sleep apnoea and life-limiting arthritis **(30)**. The Society of American Endoscopic and Gastrointestinal Surgeons also published guidance, recommending laparoscopic surgery where the necessary expertise and appropriate patient indications allowed **(31)**. BPD/DS was increasingly being performed in two stages laparoscopically, with a first operation consisting of a vertical sleeve gastrectomy (LSG). This operation removed the left side of the stomach, leaving a tubular stomach along the lesser curve. Over the last 10 years,

multiple centres noted that weight loss with LSG was comparable to RYGB and often more effective than LAGB. ASMBS updated their advice in 2012 to recommend LSG as an effective and safe primary weight loss operation **(32)**. By this time VBG had become less popular and is now not performed routinely in the UK **(21)**.

1.3.1 Evidence for bariatric surgery

The generation of national and international guidelines on bariatric surgery were underpinned by emerging evidence on its safety, efficacy in producing sustained weight loss. This was accompanied by the widening realisation that other features of the metabolic syndrome (MetS), especially diabetes, dyslipidaemia and cardiovascular pathology, were also significantly improved. Table 1.1 states one version of the criteria for diagnosis of MetS **(33)**.

Pories *et al* reported results of 608 patients undergoing gastric bypass and highlighted the durable restoration of normoglycaemia in >80% of their diabetic subgroup **(34)**. Buchwald *et al* published a meta-analysis in 2004 that provided high quality evidence of these post-operative benefits **(35)**. They reviewed 136 papers including 22094 patients. Aggregated mean weight loss for all operations was 61.2% (95% confidence interval 58.1%-64.4%), with per-procedure weight loss as follows: gastric banding 47.5% (40.7%-54.2%), gastric bypass 61.6% (56.7%-66.5%) and BPD/DS 70.1% (66.3%-73.9%). Operative mortality was very low, from 0.1% for gastric banding, 0.5% for gastric bypass and 1.1% for BPD/DS. More strikingly, the rate of resolution of important co-morbidities was extremely high - diabetes resolved or improved in 86.0%, hyperlipidemia improved in >70%, hypertension resolved or improved in 78.5% and obstructive sleep apnea resolved or improved in 83.6% of patients.

Table 1.1 International Diabetes Federation Definition of Metabolic Syndrome (taken from Alberti *et al*, Circulation 2009) (33)

Measure	Categorical Cut Points
Elevated waist circumference*	Population- and country-specific definitions
Elevated triglycerides (drug treatment for elevated triglycerides is an alternate indicator†)	≥150 mg/dL (1.7 mmol/L)
Reduced HDL-C (drug treatment for reduced HDL-C is an alternate indicator)	<40 mg/dL (1.0 mmol/L) in males; <50 mg/dL (1.3 mmol/L) in females
Elevated blood pressure (antihypertensive drug treatment in a patient with a history of hypertension is an alternate indicator)	Systolic ≥130 and/or diastolic ≥85 mm Hg
Elevated fasting glucose‡ (drug treatment of elevated glucose is an alternate indicator)	≥100 mg/dL
HDL-C indicates high-density lipoprotein cholesterol. *It is recommended that the IDF cut points be used for non-Europeans and either the IDF or AHA/NHLBI cut points used for people of European origin until more data are available. ‡Most patients with Type 2 Diabetes Mellitus will have the metabolic syndrome by the proposed criteria.	

A recent updated meta-analysis of studies published from 2003 to 2012 shows that these impressive results have proved durable **(36)**. The authors included LSG in their review and concluded that its safety profile, weight loss results and rate of resolution of co-morbidities were equivalent to RYGB. The post-surgical fall in BMI are shown in Figure 1.1. Data from this meta-analysis are presented overleaf in Table 1.3.

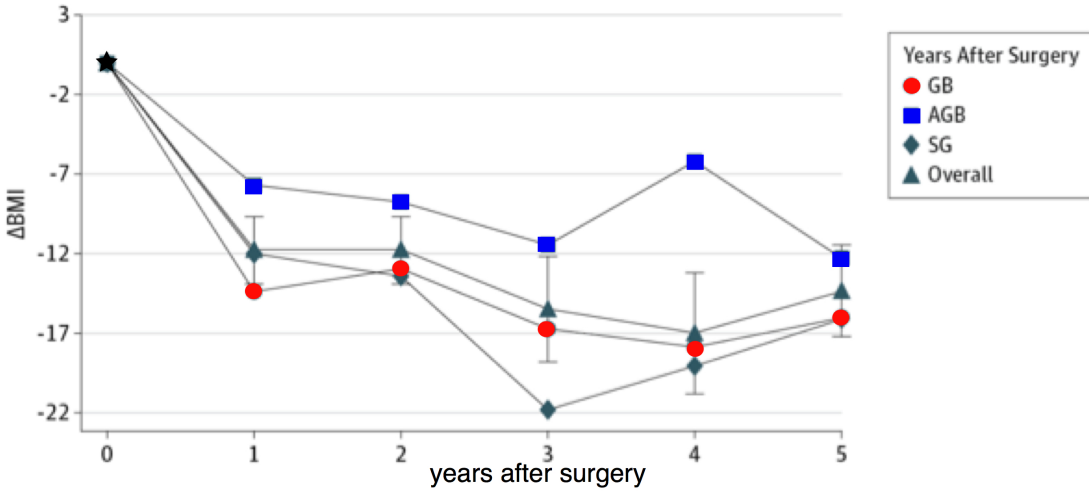


Figure 1.1: Five year data for reduction in BMI over time after RYGB, AGB and LSG (adapted from Chang *et al*, 2013) (36)

The Swedish Obese Subjects Study was a nationwide longitudinal and cross-sectional study of obese patients undergoing surgery or non-surgical intervention, followed up for up to 20 years. Various reports from this large database have indicated that bariatric surgery lead to significant reductions in co-morbidities and long-term mortality, and improvements in quality of life **(37)**.

The first report from a US national bariatric database shows similar results, with the efficacy and safety profile of LSG sitting in between LAGB and RYGB **(38)**. Morbidity and mortality data are shown in Table 1.2.

Table 1.2 Outcomes from the American Bariatric Surgery Centre Network report (based on Hutter *et al*, 2011) (38)

	LSG n=944	LAGB n=12,193	LRYGB n=14,491
30-day mortality	0.11%	0.05%	0.14%
1 year mortality	0.21%	0.08%	0.34%
30-day morbidity	5.61%	1.44%	5.91%
30-day readmission	5.40%	1.71%	6.47%
30-day reoperation	2.97%	0.92%	5.02%

Table 1.3 Weight loss and clinical outcomes after bariatric surgery (based on Chang *et al*, 2013) (36)

		GB	AGB	SG	Overall
		mean (95%CI)			
ΔBMI	Year 1				
	RCT	15 (12 - 17)	11 (7 - 14)	16 (8 - 24)	14 (12 - 16)
	OBS	14 (10 - 19)	8 (6 - 9)	12 (10 - 14)	12 (10 - 14)
	Year 2				
	RCT	14 (12 - 17)	11 (8 - 14)		13 (11 - 15)
	OBS	13 (8 - 17)	9 (7 -10)	13 (7 - 20)	12 (10 - 14)
	Year 5				
	RCT		11 (5 - 28)		
	OBS	16 (11 - 21)	12 (8 - 17)	16 (4 - 28)	14 (11 - 17)
%EBWL	Year 1				
	RCT	72 (65 - 80)	33 (23 - 44)	70 (41 - 98)	60 (51 - 69)
	OBS	63 (54 - 72)	34 (34 - 35)	51 (44 - 59)	46 (44 - 48)
	Year 2				
	RCT	74 (66 - 83)	54 (33 - 75)		71 (63 - 79)
	OBS	80 (66 - 94)	52 (49 - 56)	47 (43 - 51)	64 (55 - 72)
	Year 5				
	RCT		41.6 (-9 - 93)		
	OBS	65 (44 - 86)	57 (47 - 67)		62 (59 - 66)
Mortality ≤30days	RCT	0.08 (0.01 - 0.30)	0.11 (0.01 - 0.50)	0.50 (0.01 - 3.88)	0.08 (0.01 - 0.24)
	OBS	0.38 (0.22 - 0.59)	0.07 (0.02 - 0.12)	0.29 0.11 - 0.63)	0.22 (0.014 - 0.31)
Morbidity	RCT	21 (12 - 33)	13 (5 - 26)	13 (0.7 - 44)	17 (11 - 23)
	OBS	12 (7 - 17)	8 (3.9 - 13)	9 (6 - 13)	10 (7 - 13)
Diabetes Remission	RCT	95 (88 - 99)	74 (36 - 96)		92 (85 (97)
	OBS	93 (85 - 97)	68 (50 - 83)	86 (73 - 94)	86 (78 - 92)
Hypertension	RCT	81 (68 - 92)	54 (13 - 90)		75 (62 - 86)
	OBS	78 (64 - 89)	64 (52 - 75)	82 (68 - -92)	74 (67 - 81)
Dyslipidemia	RCT	80 (62 - 94)	40 (4.7 - 87)		76 (56 - 91)
	OBS	63 (41 - 82)	61 (49 - 72)	83 (63 - 95)	68 (58 - 77)
OSA	RCT	95 (84 (100)	94 (49 - 100)		96 (87 - 100)
	OBS	95 (86 - 99)	71 (48 - 89)	91 (80 - 97)	90 (81 - 95)

ΔBMI – reduction in BMI from baseline, kg/m^2, %EBWL – percentage of excess body weight lost from baseline; excess body weight = actual body weight – ideal body weight (height^2 X 25 (ideal BMI), RCT= randomised control trial data, OBS=data from observational studies, OSA=obstructive sleep apnoea

1.3.2 Common operations for weight loss

The main four operations performed for weight loss in the UK at present are: RYGB, LAGB, BPD/DS and LSG. Schematic diagrams of these operations are given in Figure 1.2. A number of newer innovations are increasingly being trialled but these are outwith the scope of this review **(39)**.

1.3.2.1 Roux-en-Y Gastric Bypass

This is the most commonly performed procedure, >50% of all weight loss surgery **(21)**. Although there are multiple published technical adjustments, broadly the operation includes **(40)**: fashioning of the most proximal part of the stomach into a pouch of 15-30ml^3 using staplers, construction of a Roux loop of jejunum, with the proximal end approximately 70-100cm distal to the pylorus disconnected, brought up and anastomosed to the gastric pouch, and the distal end of the biliopancreatic limb anastomosed approximately 100-150cm from the gastric anastomosis, forming a common channel. Thus dietary intake is restricted by a small gastric pouch. Food bypasses the rest of the stomach and duodenum to pass down the alimentary limb, whereupon 100cm distally it mixes with gastric and pancreaticobiliary secretions and is absorbed in the small bowel.

RYGB was presumed to work by restricting intake mechanically due to small gastric pouch size and behaviourally, as patients sought to avoid unpleasant side effects like discomfort from early satiety, so called “plugging” as food gets held up in the gastric pouch, and “dumping syndrome” which occurs due to rapid transit of food into the small bowel causing flushing, nausea and diarrhoea **(41)**. The rapid resolution of resolution of diabetes mellitus within weeks of surgery gave rise to interest in the hormonal actions of gastric bypass (see also Table 1.6) **(34)**. It is now recognised that weight loss and resolution of co-morbidities occur through a combination of different mechanisms, including but not limited to changes in gut hormone levels affecting insulin release, hunger and satiety, changes in taste preference and adaptations in energy homeostasis and regulation **(42)**. Intestinal is not significant after gastric bypass **(43)**.

1.3.2.2 Adjustable Gastric Banding

There are various gastric banding devices commercially available and the procedure involves placement of a silastic inflatable band around the cardia of the stomach just below the oesophagogastric junction. This acts as a constant restrictive presence and can be tightened/loosened by instillation/removal of fluid via a subcutaneous port. The mode of action was thought to be restrictive although transit and manometric studies now suggest that the band acts neurally, possibly by compressing the vagus nerve at the oesophageal hiatus, to activate the satiety mechanism **(44)**. These neural changes probably contribute to behavioural changes and result in a reduction in energy intake and weight loss. LAGB remains the safest procedure in the short term, with 30 day complication rates of 2.3-6.1% and mortality of <0.1% **(45)**. There are concerns of long term safety. At least 1% of patients will experience band erosion, with erosion occurring in mean 4.9% of cases in one meta-analysis **(46)**. This may be an underestimate as in one study of 665 patients treated over 12 years, 21% of bands had to be removed during the study period **(47)**.

1.3.2.3 Biliopancreatic Diversion/Duodenal Switch

This involves two stages, that can be performed separately or in one operation: creation of a sleeve gastrectomy by removing 90% of the stomach and a duodeno-ileal bypass, which involves transecting the first part of the duodenum and the ileum and anastomosing the jejunum proximally to the pylorus and distally to the ileum just 100 cm proximal to the ileocaecal valve. The very short common channel of 100cm or less causes profound malabsorption **(43)**. Average excess weight loss after BPD-DS is >70%, which is greater than the other operations discussed, with more nutritional complications **(35)**. Operative mortality and complication rates are higher **(35)** and this operation is less widely performed **(21)**.

1.2.3.4 Laparoscopic Sleeve Gastrectomy

Using staplers, the left part of the stomach is excised starting from a point on the greater curve approximately 5 cm proximal to the pylorus (to preserve the antrum), running in a straight line cephalad towards the angle of His **(48)**. This leaves a tubular stomach remnant, whose width is calibrated during stapling using a bougie **(32)**. Staple line reinforcement is used by some surgeons to decrease the risk of leakage and bleeding **(49)**. Various calibre bougies are used from 32 to 60 French guage depending on surgeon preference **(50)**. The narrower the calibre is, the greater the weight loss expected although some authors are also concerned about increasing risks of staple line leakage and bleeding **(49, 51)**.

LSG was first performed as the first stage procedure of BPD-DS in very high BMI patients (>60kg/m^2), to reduce the risk of complications and effect some initial weight loss before proceeding with the more risky second stage of surgery **(32)**. However, the results of LSG alone were comparable to RYGB, with similar rates of resolution of co-morbidities **(52, 53)**. The proportion of patients undergoing LSG as a primary surgical treatment has increased over the last 6 years **(50, 54)**. Long-term weight loss results are also emerging, confirming its efficacy is durable **(38)**. Eid *et al* reported 48% excess weight loss after mean follow-up of 73 months in the largest series to date of 69 patients **(55)**.

Although LSG was seen primarily as a restrictive procedure with reduction in both calibre and total volume of the stomach, it is apparent that other mechanisms may contribute to weight loss and resolution of diabetes **(56)**. These include: resection of the fundus reduces ghrelin production changing hunger and satiety **(57)**, increase in gastric emptying may affect nutrient delivery to the small bowel **(57)**, adaptations to gut hormone release affect glycaemic control **(58)**, and post-operative circulating cytokine profiles are similar to RYGB **(59)**.

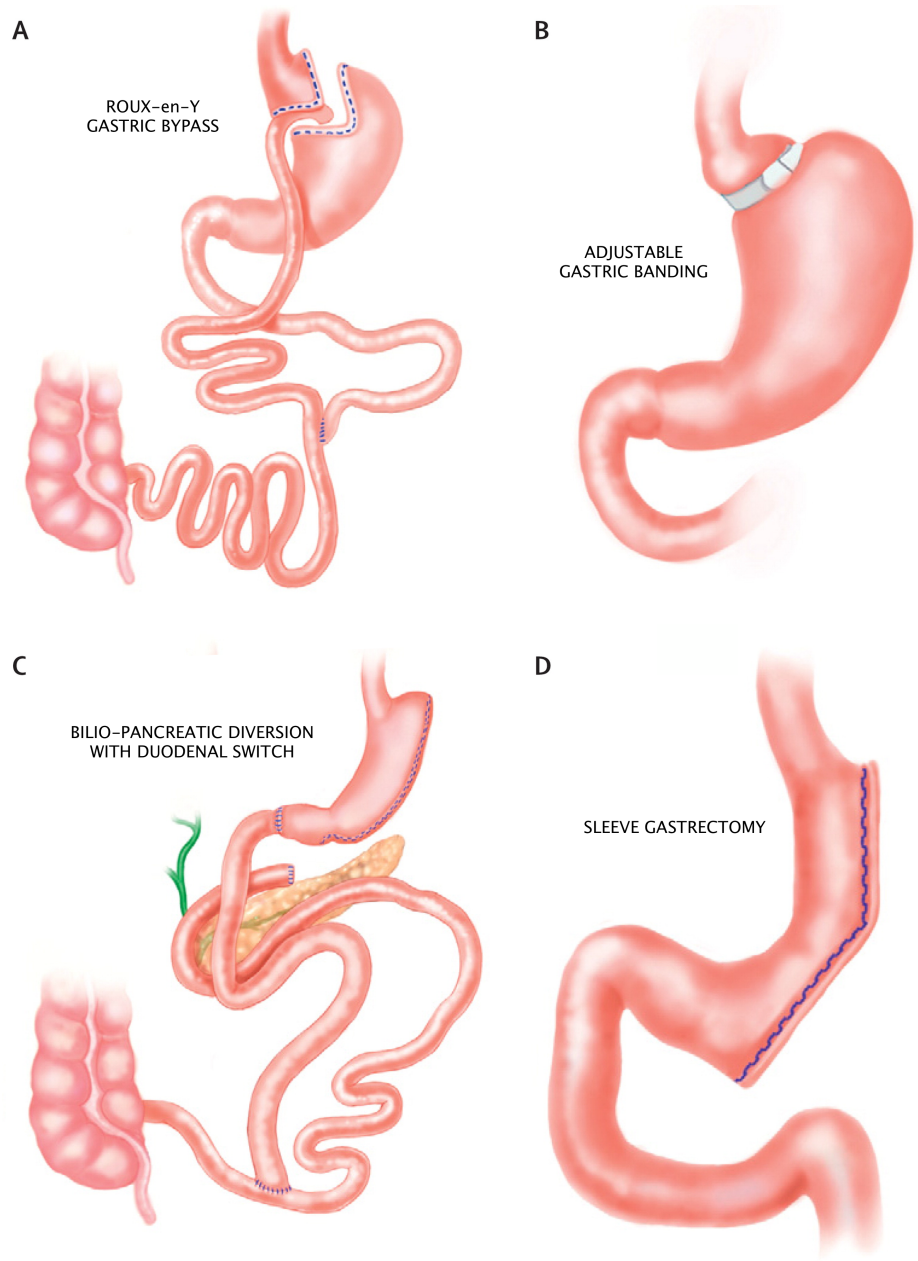


Figure 1.2 Schematic representations of four main bariatric surgery operations performed in United Kingdom, RYGB, AGB, BPD/DS, LSG (adapted from Dixon *et al*, 2012) (60)

1.3 Obesity and Inflammation

The link between obesity and a low-grade chronic inflammatory state was highlighted by a number of studies showing that serum C-reactive protein (CRP) levels were elevated in obesity and correlated with a higher risk of cardiovascular events **(61)**. CRP is a so-called acute phase protein secreted by the liver in response to inflammatory, infective and other toxic insults. It has a role in innate immunity, activating complement and opsonising pathogens **(62)**. It has an important clinical use to monitor the response to and resolution of such insults. Interleukin-6 (IL-6) is another pro-inflammatory cytokine released acutely and increases secretion of CRP **(63)**. Visser *et al* demonstrated in a study of over 16000 people that CRP levels are higher in overweight and obese individuals compared with lean controls **(64)**. Smaller scale studies have shown increased expression of IL-6 is also associated with obesity and is linked to increased adipose tissue mass, and such an association with adiposity is seen with other pro-inflammatory cytokines including tumour necrosis factor (TNF) α **(61)**. Weight loss through diet, exercise or bariatric surgery is clearly associated with diminution of circulating pro-inflammatory cytokines, indicating proof of the concept **(65)**. The elevation in pro-inflammatory cytokines are a causative link between obesity, increased adipose tissue, hyperlipidemia and atherosclerotic cardiovascular disease **(66)**.

1.3.1 Adipose tissue is the source of inflammation

Adipose tissue in obese patients has increased levels of macrophages. The number and activity of these macrophages correlate with body mass index (BMI) and size of adipose cell, with a corresponding correlation with expression of TNFα and IL-6 **(67)**. Macrophage expression of pro-inflammatory cytokines leads to increased production of other cytokines from adipose tissue **(68)**. Insulin resistance may also be an effect of this pro-inflammatory state. Xu *et al* used a mouse model to demonstrate that pro-inflammatory genes were upregulated in the white adipose tissue of obese mice compared with lean controls and that this upregulation occurred mostly in macrophages **(69)**. They also showed that administration of an insulin-sensitising drug, Rosiglitazone, downregulates the pro-inflammatory genes, suggesting that interplay between macrophages and adipocytes may

generate insulin resistance itself. Their hypothetical model of the development of insulin resistance is illustrated in Figure 1.3.

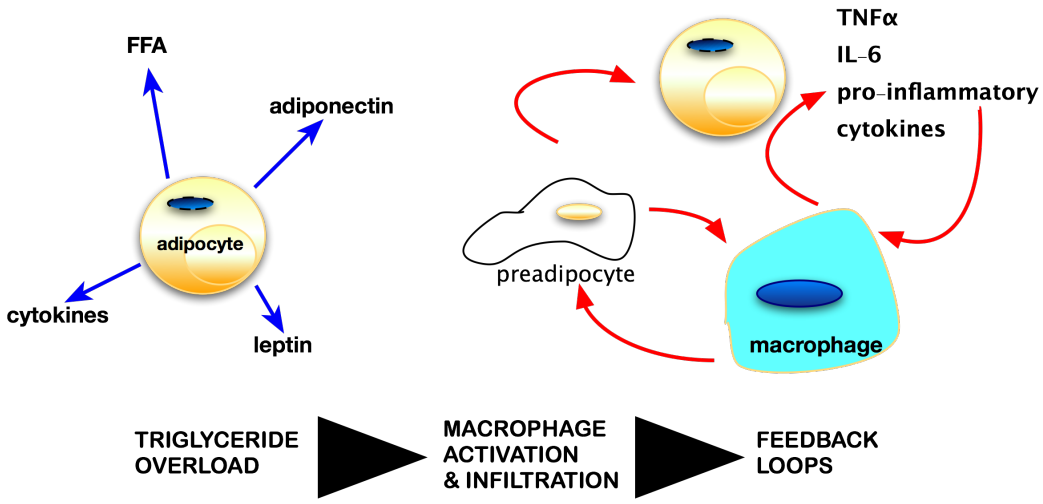


Figure 1.3 The development of insulin resistance through triglyceride overload and chronic inflammation (adapted from Xu *et al*, 2003) (69)

Beyond a certain, as yet unknown threshold, accumulation of intracellular lipid in adipocytes leads to an inflammatory response, with local release of cytokines and chemokines, attracting and activating macrophages, and spillover of free fatty acids (FFA) into the circulation. These activated macrophages also secrete inflammatory mediators. These may affect preadipocytes and adipocytes, inducing insulin resistance. The whole process becomes a vicious cycle, amplifying the inflammatory response and eventually causes systemic insulin resistance.

1.3.2 Obesity and Oxidative Stress

Obesity is associated with oxidative stress **(70)**. Oxidative stress is the imbalance between oxidant and antioxidant factors within cells, which leads to cellular damage and inflammation **(71)**. Oxidant molecules, also known as reactive oxygen species (ROS) including free radicals such as superoxide ($O2^{\bullet -}$), peroxide ($O2^{\bullet -2}$) and hydroxyl radical ($^{\bullet}OH$), are produced as byproducts in the mitochondria during the oxidation of glucose or free fatty acids (FFA), which is an essential process in energy metabolism. ROS are potentially toxic and may combine with lipids and proteins. Lipid peroxidation may alter the activity of receptors, decrease the activity of membrane bound enzymes or alter cell permeability. Protein

oxidation leads to formation of protein carbonyls (PC) which again may affect enzyme activity, protein-protein, lipid-protein and lipid-lipid interactions, including changes in gene expression **(72)**. A complex antioxidant system exists to neutralise ROS, including both non-enzymatic and enzymatic processes. Non-enzymatic antioxidants include vitamins C and E and glutathione, which act by trapping and reducing free radicals, neutralising their effects. The enzymatic antioxidant system includes superoxide dismutase (SOD), catalase, glutathione reductase and peroxidases (GPX), which degrade ROS into less harmful molecules like water and oxygen **(73)**. Olusi compared the concentrations of markers of lipid peroxidation and antioxidant enzymes in healthy and obese populations, and found that BMI positively correlates with circulating malondialdehyde (MDA, a marker of lipid peroxidation) and negatively correlates with SOD and GPX **(74)**.

Furukawa *et al* demonstrated that markers of oxidative stress correlated with increasing adipose tissue mass. In a cell model, the same authors showed that oxidative stress may be mediated by increased levels of free fatty acids **(70)**. They found increased levels of ROS in the adipose tissue of obese mice, with increased expression of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), a key enzyme in mitochondrial energy metabolism and an important source of ROS. They also found reduced expression of antioxidants **(70)**.

Pro-inflammatory cytokines are found in association with increased oxidative stress and many of the same factors lead to both sets of findings **(75)**. The corollary is that inflammation and oxidative stress co-exist and together cause obesity-related pathology and insulin resistance. The interplay between oxidative stress and inflammatory response is depicted in Figure 1.4.

1.3.3 Inflammation, Obesity and Insulin resistance

Obesity-related pro-inflammatory changes are now believed to lead to insulin resistance. In a longitudinal cohort study of almost 6000 patients, elevated CRP at baseline whilst still normoglycaemic was found to be a very strong predictor of future diabetes mellitus **(76)**. When comparing obese patients with insulin-resistance to normoglycaemic obese patients, a number of metabolic differences

are seen. Obese insulin resistant individuals have comparatively higher levels of immune cells within their adipose depots, higher measures of oxidative stress and higher levels of cytokines **(68)**.

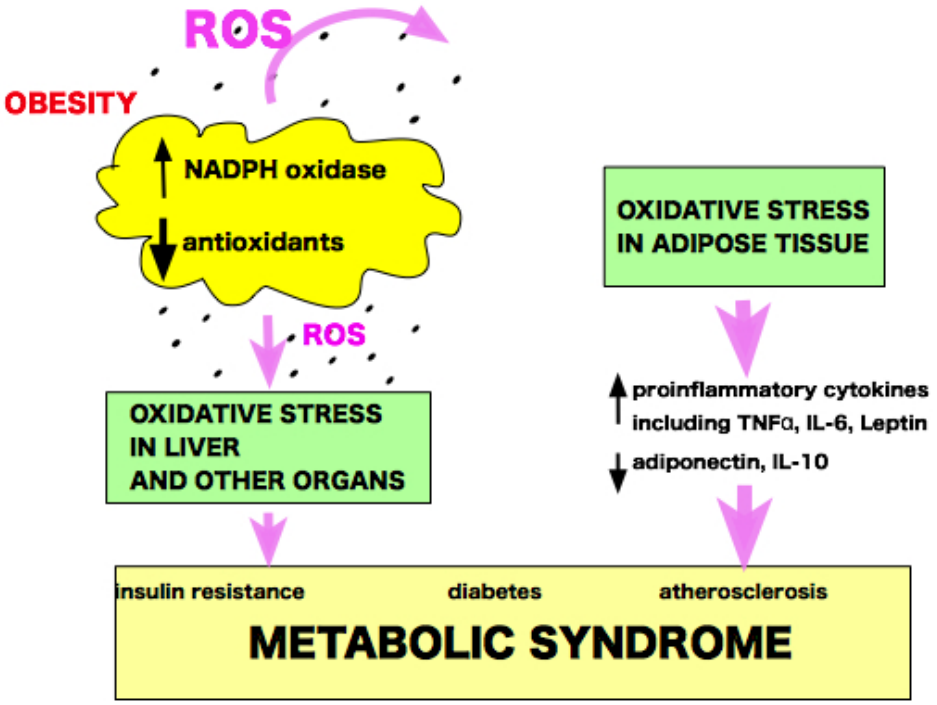


Figure 1.4 Reactive oxygen species generated in adipose tissue promulgate the induction of oxidative stress remotely, leading to metabolic syndrome (adapted from Furukawa *et al*, 2004) (70)

This is thought to be related to combination of ischaemia within the adipocytes due to their large size, impairment of the endoplasmic reticulum leading to changes in FFA, glucose and protein metabolism and the changes in the cytokine milieu and oxidative stress discussed above **(75)**. The final common pathway relating these changes to insulin resistance is not yet clear. Xu *et al* showed that adenosine monophosphate-activated protein kinase (AMP-kinase) activity in fat depots are lower in obese insulin-resistant patients than in the insulin-sensitive group, along with higher expression of pro-inflammatory genes and worse markers of oxidative stress **(77)**. AMP-kinase dysregulation leads to lipid accumulation, changes in FFA metabolism and is associated with insulin-resistance **(78)**.

There are an ever-increasing number of different cytokines and other potential ligands of interest. A long but not exhaustive list is given in Table 1.4 of mediators

isolated from adipose tissue, most of which have not been extensively studied as yet **(79)**. The most important and well studied cytokines – the adipokines - leptin, adiponectin and resistin, TNFα and IL-6 – are discussed in the introduction to Chapter 3, as these are the ones evaluated in this thesis.

Table 1.4 Thirty Seven Mediators isolated from adipocytes that are affected by obesity (taken from Fain, 2010) (79)

Name of Adipokine	Change in circulating levels in obesity
ACE (angiotensin converting enzyme)	Elevated
Adiponectin	Lower
Adipsin	Elevated
Amyloid A	Elevated
Cathepsin S	Elevated
Cluster of differentiation 14	No change
CRP	Elevated
Fatty acid binding protein4	Elevated
Glutathione peroxidase 3	Lower
Haptoglobin	Elevated
Hepatocyte growth factor	Elevated
Intracellular adhesion molecule 1	Elevated
IL-10	Elevated
IL-18	Elevated
IL-1Ra	Elevated
IL-1β	No data
IL-6	Elevated
IL-8	Elevated
Leptin	Elevated
Lipocalin-2	No change
Lipoprotein lipase	No change
Monocyte chemotactic protein 1	Elevated
Migration inhibitor factor	Elevated
Nerve growth factor	Elevated
Osteoprotegerin	No change
Plasminogen activator 1	Elevated
Prostaglandin E2	No data
Chemokine ligand 5	No change
Resistin	No change
Soluble TNF Receptor II	Elevated
Transforming growth factor- β 1	Elevated
TNF α	Elevated
Vascular cell adhesion molecule 1	Elevated
Vascular endothelial growth factor	Elevated
VEGF receptor	No change
Visfatin	No change
Zinc-α2-glycoprotein	No change

IL- interleukin, adipokines are in alphabetical order

1.4 The effects of bariatric surgery

1.4.1 Bariatric Surgery and Insulin Resistance/Diabetes

The various modes of bariatric surgery are all associated with significant weight loss, along with high rates of resolution or improvement in type 2 diabetes (T2DM). In a meta-analysis of over 4000 patients, diabetes resolved in 78% of patients, with higher rates after malabsorptive surgery (BPD/DS, see Table 1.5) **(80)**. In 5 studies of sleeve Gastrectomy including 123 patients, 67% achieved resolution of T2DM at 12 months, with an excess weight loss of 67% **(81)**.

Table 1.5: Resolution of diabetes after bariatric surgery – data from a meta-analysis (Buchwald *et al*, 2009) (80)

	Total	Gastric Banding	Gastroplasty	Gastric Bypass	BPD-DS
% EBWL	55.9	46.2	55.5	59.7	63.6
% Resolved overall	78.1	56.7	79.7	80.3	95.1
% Resolved <2 years	80.3	55	81.4	81.6	94
% Resolved >2 years	74.6	58.3	77.5	70.9	95.9

Note: Resolution of diabetes defined as 'off diabetic medications with HbA1c <6%'

1.4.2 How does bariatric surgery improve or resolve diabetes?

The mechanisms by which resolution of diabetes occurs are hotly debated and outwith the scope of this review. Randomised trials comparing weight loss and T2DM resolution between bariatric surgery and diet and exercise programmes have all favoured surgical treatment. The mean difference in weight loss between surgery and non-surgical controls was −26 kg (95% confidence interval −31 to −21, $p<0.001$) in 10 studies including 658 patients **(82)**. Although heterogeneity between studies was high, pooled analysis of 4 studies including 252 patients showed that the relative risk to achieve diabetes remission was at least 5.3 times and perhaps up to 22 times higher in the surgical group **(82)**. RYGB and BPD/DS affect insulin resistance within days of operation, before significant weight loss occurs, with an immediate effect greater on fasting insulin and glucose than that seen in patients on very low calorie diets **(83)**.

The main theories postulated to account for the rapidity of surgical resolution are based on intestinal hormonal regulation of insulin. As well as the direct effect of circulating glucose concentration on insulin release from pancreatic beta cells, a number of other hormones are thought to exert an effect. These hormones have collectively been termed "incretins" and include glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP). Post-RYGB and BPD/DS, incretin levels are much higher after a glucose challenge or test meal, which correlate with increases in insulin levels **(84)**. To account for this incretin effect, two competing hypotheses are described: the foregut hypothesis says that bypassing the duodenum and proximal jejunum reduces release of inhibitors of the incretin hormones; the hindgut hypothesis suggests that bypass of the proximal intestine leads to greater than normal levels of nutrients to come into contact with the distal small bowel, stimulating a greater incretin effect **(85)**. The other putative mechanisms also depend on the altered release of other gut hormones, including peptide YY (PYY) which suppresses appetite and ghrelin, involved in regulation of satiety, hunger and body weight **(86)**. The changes in gut hormones after RYGB are briefly summarised in Table 1.6.

Table 1.6: Gut Hormone changes after RYGB and LSG (based on a review by Michalakis and Le Roux, 2012) (87) (88). Gut hormone changes after LSG are similar to RYGB but in comparative studies, are generally less marked.

Hormone	Action	Change after RYGB and LSG
PYY	Inhibits of gastric emptying	Levels after meal are increased after surgery compared to any of the weight control groups (lean, overweight, obese)
	Inhibits gallbladder contraction and secretion of pancreatic and gastric secretions	Levels are increased more than with other types of surgery
	Reduces expression and secretion of NPY (orexigenic) in brain	
	Rises postprandially	
Ghrelin	Levels rise with fasting and fall after a meal	Levels reduced after RYGB
	Ghrelin injection activates arcuate NPY/agouti-related peptide neurons causing increased appetite	restrictive gastric surgery (eg AGB) without the RYGB construction does not lower ghrelin levels, although Ghrelin is reduced after LSG
	NPY/AgRP knock-out mice lacked orexigenic response to ghrelin administration	
OXM	Reduces weight, increases satiety, reduces body fat	
	Increases energy expenditure	
GIP	Increased levels in obese patients	
	Promotes energy storage	
	Lipolytic actions and anabolic actions on adipocytes	
GLP-1	Delays gastric emptying and inhibits gastric acid secretion	Increased levels after RYGB and LSG (increase is smaller)
	Enhances insulin release and reduces glucagon secretion	Higher levels after RYGB than LSG, but both are higher than AGB
	– Reduces food intake and induces satiety	Higher levels after RYGB and LSG than patients with low calorie diet

PYY – peptide YY, OXM – oxyntomodulin, GIP - Glucose-dependent insulinotropic polypeptide (a “classic” incretin), GLP-1 – glucagon-like peptide 1 (a “classic” incretin)

1.4.3 Bariatric Surgery and Inflammation

Dietary interventions and physical exercise programmes can lead to significant weight loss and there have been many studies showing short-term reductions in inflammatory markers **(89)**. Forsythe *et al* neatly summarise 65 studies measuring various combinations of CRP, TNFα, IL-6, Leptin and Adiponectin following interventions involving diets, physical activity, diets and physical activity combined and weight loss surgery. Of dietary interventions only, 7 of 23 published studies showed no significant falls in inflammatory markers. 17 of 18 studies of combined diet and exercise interventions showed significant reduction in weight and inflammatory markers. As expected, studies where weight loss and reduction of adipose tissue mass were greater were associated with a greater reduction in inflammatory markers. In contrast, weight loss surgery (25 studies quoted) was associated with much greater weight loss and thus a greater reduction in inflammatory markers **(89)**.

1.4.3.1 The effect of Bariatric Surgery on Pro-inflammatory cytokines

In a systematic review of 48 studies, bariatric surgery was shown to significantly reduce CRP from 1 month post-operation onwards, including studies with >12 month follow up. On meta-regression, fall in BMI was the only significant predictor of fall in CRP. IL-6 was seen to fall significantly after 6 months and this reduction was also sustained in studies of >12months duration. The percentage reduction in TNFα following surgery was not significant **(90)**. The values of CRP, IL-6 and TNFα are tabulated in Table 1.7.

CRP production by the liver is stimulated by both IL-6 and TNFα and all three markers are known to be higher in obesity and insulin resistance **(91)**. Taking the corresponding falls in CRP and IL-6 together with post-operative weight loss and improvement in insulin resistance, these studies corroborate the various cell and animal models highlighting their role in obesity-related chronic inflammation **(92)**. Cancello *et al* showed that macrophage infiltration is reduced in subcutaneous fat after bariatric surgery, along with a decrease in expression of genes involved in chemoattraction and inflammation **(93)**. Greco *et al* found reduced intramuscular lipid deposition, which correlated with weight loss and

improvement in insulin resistance, indicating that peripheral adipose and lipid depots are reduced **(94)**.

Table 1.7 Percentage reduction in CRP, TNFα and IL-6 from a meta-analysis of 48 studies (based on Rao, 2012) (90)

Parameter	Length of Follow-up	k	n	Weighted pre-op mean	%change at follow-up ±SEM
CRP	Maximum	42	2877	10.1879	−65.8 ±4 **
	1 month	6	134	16.154	−34.3 ±8 **
	3 months	3	68	10.3	−54.6 ±14 **
	6 months	20	816	10.7968	−54.0 ±5 **
	12 months	10	1738	9.8782	−81.0 ±13 **
	>12 months	8	326	9.7289	−73.1 ±5 **
TNF	Maximum	13	360	8.2825	−1.0 ±2
	6 months	4	150	5.3072	−1.3 ±5
	12 months	4	160	6.6565	−1.3 ±3
	>12 months	4	185	6.8177	−2.9 ±3
IL-6	Max follow-up	16	625	5.1464	−27.4 ±3 **
	1month	3	66	3.7573	10.9 ±15
	6 months	8	416	4.7109	−21.5 ±3 **
	12 months	4	177	4.5745	−36.2 **
	>12 months	4	185	5.1738	−33.7 ±4 **

**p<0.001; n – number of patients, k – number of studies

TNFα is also heavily implicated in the development of insulin resistance, with evidence from cell models, knockout mice, human studies with exogenous administration of TNFα or inhibition by antagonists **(90, 95, 96)**. However it is

also produced in the liver, where levels are higher in NASH and fibrosis, which are more likely to persist after surgery even after depletion of fat depots. Ghaemi *et al* measured CK-18 and inflammatory markers in 44 patients with NAFLD undergoing a diet and exercise programme **(97)**. After 6months, patients who had lost >5% body weight had significantly reduced liver function tests and CK-18 but no significant differences in TNFα and IL-6, suggesting that a longer time period with a greater degree of weight loss is required before reductions in inflammatory markers are seen. It is also not clear whether circulating TNFα concentration reflects the local concentration in the visceral and peripheral adipose tissue and liver, with some inflammatory markers having higher concentrations in the portal vein than in the systemic circulation **(91)**. In comparison, TNFα also has a shorter half life than IL-6 and CRP is a very stable molecule **(98)**. The heterogeneity of results seen may reflect variation in the methods of collection, storage and measurement of less stable markers, including TNFα.

1.4.3.2 The effect of bariatric surgery on adipocytokines

With increasing evidence for their role in the pathogenesis of insulin resistance, diabetes and steatohepatitis, adipokines including adiponectin, leptin and resistin, have been widely studied in the bariatric surgery population. Illan-Gomez *et al* measured adiponectin in 53 patients undergoing RYGB over a year, and found that levels were much lower than lean controls pre-operatively, and rose over the course of the study to almost double from 5.82μg/ml ±SD 2.93 to 10.97±5.56 **(99)**. The rise in adiponectin was accompanied by a fall in IL-6 and CRP, along with reductions in BMI and insulin resistance. Interestingly, adiponectin levels at baseline and 12 months did not correlate significantly with BMI or HOMA-IR but IL-6 did at 12months. In contrast, Auguet *et al* found that the adiponectin rise following LSG and RYGB in 30 patients plateaued at 6 months and negatively correlated with insulin resistance but did not correlate with the fall in blood lipid concentrations **(100)**. They also studied visfatin concentration, a pro-inflammatory adipocytokine, which fell after 12 months. In contrast to other similar studies, they measured circulating TNF receptors I and II, which also fell in concentration after 6 and 12 months. The same group also measured leptin and chemerin in the same cohort, showing significant falls over 12months **(101)**.

Miller *et al* measured adiponectin and other inflammatory markers at baseline, 3 weeks, 3 months and 6 months after RYGB (see Figure 1.5). They found that the post-operative rise in adiponectin is already significant at 3 weeks and no further significant rises occur **(102)**. In contrast, leptin levels fell significantly at each timepoint. The fall in CRP only became significant at 6 months. They concluded that leptin levels are linked to adipose mass and correlate with BMI, unlike adiponectin. The authors also comment on the difficulty in measuring TNFα levels and measured circulating TNF receptor concentrations instead as these are more stable.

Swarbrick *et al* measured adipokines in combination with different pancreatic enzyme concentrations, including pancreatic polypeptide and glucagon, in 19 patients undergoing RYGB **(103)**. Pancreatic enzyme levels fell over multiple timepoints over 12 months along with falls in pro-inflammatory cytokines and improvements in insulin resistance.

Ianelli *et al* compared changes in CRP between 12 patients undergoing RYGB and 10 patients LSG at 6 and 12 months after surgery **(104)**. They found larger reductions in CRP in the RYGB group, which correlated with greater falls in BMI and improvements in insulin resistance. In a randomised trial comparing LSG and RYGB, there were no significant differences between procedures in the respective post-operative fall and rise of leptin and adiponectin over 12 months (see Figure 1.6) **(59)**. These results indicate that production of these adipocytokines is not affected by different methods of bariatric surgery and are unlikely to be under the direct influence of gut hormones, which are affected differently by LSG and RYGB.

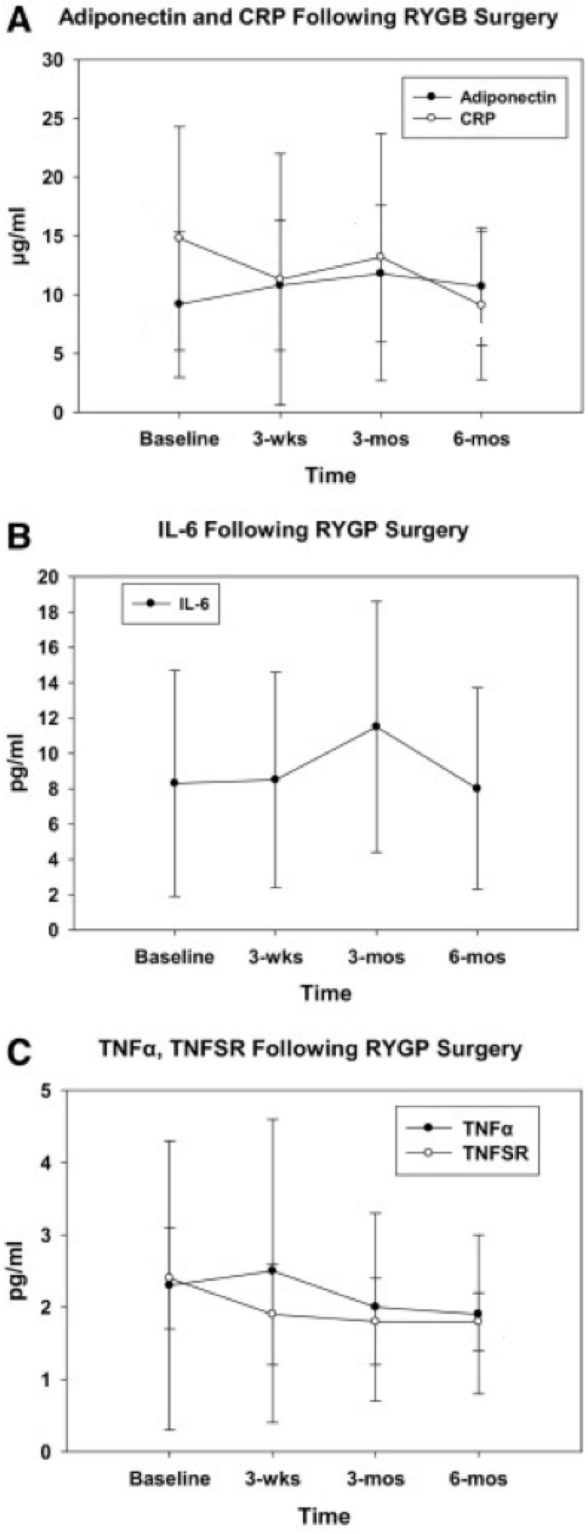


Figure 1.5 Changes in Inflammatory markers and cytokines over the first 6 months after RYGB (adapted from Miller *et al*, 2011) (102)

There are a number of similar longitudinal cohort studies, which confirm that bariatric surgery is associated with falls in pro-inflammatory markers including CRP, IL-6, leptin and visfatin, and a rise in adiponectin and IL-10. The effect on TNFα is unclear although data from studies measuring circulating TNF receptors, which are in proportion to TNFα concentration, show a fall in levels. It is also clear that there is a wide heterogeneity in actual measured levels, with large standard deviations and much study-to-study variation, perhaps reflecting both the heterogeneity of levels within this population and technical difficulties relating to sample collection and measurement.

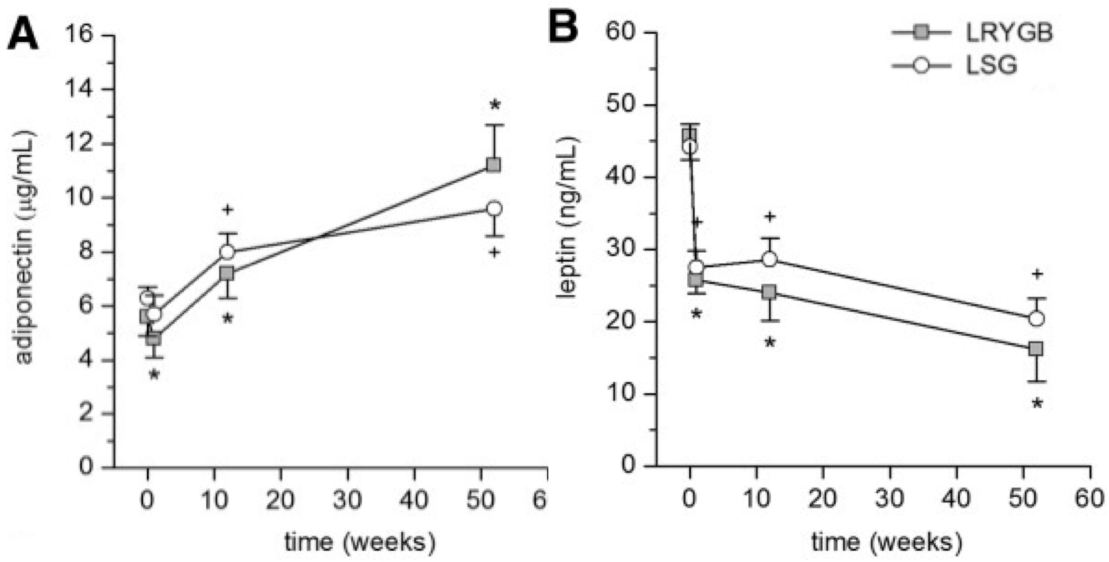


Figure 1.6 Adiponectin (A) and Leptin (B) levels after RYGB and LSG – results from a randomised trial (adapted from Woelnerhanssen *et al*, 2011) (59)
Changes in adipocytokines were similar after both types of surgery.

1.4.4 Bariatric Surgery and Oxidative Stress

Given the link between obesity and oxidative stress, there has been much interest in the effect of weight loss **(105)**. Wycherley *et al* examined the effect of a 12 week moderate diet, with and without an exercise programme on 29 patients **(106)**. They found that an 8-9% reduction in body weight was associated with significant reductions in malondialdehyde (MDA) and improvements in glycaemic control. Melissas *et al* used intragastric balloons to effect weight loss in 16 patients and found that corrected total antioxidant capacity (cTAC) increased by 33% after 6 months, but were still 15% less than non-obese controls **(107)**.

1.4.4.1 Circulating markers of oxidative stress are reduced by surgery

In contrast to non-surgical intervention studies, bariatric surgery is associated with much more significant and prolonged weight loss **(89)**. Uzun *et al* showed that mean 20-22% excess weight loss (%EWL) is associated with approximately 20% reduction in MDA 6 months after gastric banding in 40 patients. **(108)**. Endothelial cell dysfunction and changes in nitric oxide levels are also implicated in obesity-induced oxidative stress. Lin *et al* measured the combined nitrite and nitrate (NOx) in serum, along with 8-iso-prostaglandin F2α (8-iso-PGF2α) level which is another oxidative stress marker, 3-6 months after weight loss surgery **(109)**. They found significant reductions in both markers, which correlated with weight loss and reductions in HOMA-IR and triglyceride levels. The markers did not correlate with concomitant reductions in CRP or increase in adiponectin, suggesting that oxidative stress was related to glycaemic control and dyslipidaemia rather than directly to inflammatory status. Cabrera *et al* demonstrated no association between HOMA-IR and reductions in oxidative stress **(110)**. They comprehensively measured oxidative stress in 20 patients 12 months after RYGB, using MDA, glutathione and reduced glutathione, SOD and catalase as measures of antioxidant defense and total radical antioxidant parameter (TRAP), which is a measure of total antioxidant levels. They found significant increases in all antioxidant defense markers (catalase, SOD, TRAP, glutathione) and reductions in MDA and reduced glutathione. However, these changes from baseline did not correlate with HOMA-IR or CRP. Murri *et al* measured various markers at 15, 30, 45 and 90 days after BPD and found that oxidative stress initially was greater after surgery before falling at 90 days **(111)**.

Although these changes were accompanied by improvement in insulin resistance and adipocytokines levels, they did not examine any correlations with oxidative stress. Marfella *et al* used nitrotyrosine as a marker of oxidative stress-related protein damage after BPD and found that mean nitrotyrosine levels had fallen by almost 50% 1 month after BPD **(112)**. This was accompanied by a significant improvement in glycaemic control, measured using both fasting and post-prandial studies. They concluded that improved glycaemic control, especially reduction in hyperglycaemic episodes, is the most important factor in reducing oxidative stress.

A number of other studies have also shown that various markers of oxidative stress fall or improve after bariatric surgery **(113-117)**. Although these studies are fairly concordant in their findings, it should be noted that they are all small scale studies with follow up limited to 12 months or less. At present there are no long term studies of >24 months of oxidative stress markers. There is also little agreement on standardised measures of oxidative stress. Individual markers are discussed in more detail in Chapter 3.

1.4.4.2 Tissue markers of oxidative stress after bariatric surgery

As well as multiple reports of longitudinal studies of circulating markers, more recently further evidence has emerged from studies of tissue. Segments of small subcutaneous arteries together with surrounding peripheral fat have been studied before and 6 months after RYGB to evaluate the changes in contractile function **(118)**. Perivascular adipose tissue (PVAT) is locally active, secreting vasoactive peptides and is subject to the same hormonal mechanisms as other fat depots. The authors demonstrated that PVAT from obese subjects has less vasodilatory activity then lean controls and, 6 months after RYGB, the PVAT's anticontractile activity on *in vitro* arterioles had been restored, along with immunohistochemistry evidence of decreased macrophage infiltration and increased local adiponectin expression and significantly greater local nitric oxide activity. Along with an effect on PVAT's vascular activities, surgery is also associated with remodeling of small arteries, leading to overall improvements in microvascular structure and function **(119-121)**.

CHAPTER 2

FATTY LIVER DISEASE

2.1 Aims of Chapter

The pathophysiology and consequences of non-alcoholic fatty liver disease (NAFLD) are described, including the development of non-alcoholic steatohepatitis (NASH). An overview of the diagnosis of NAFLD and NASH is given. The chapter then details the phenomenon of ischaemia-reperfusion injury (IRI) and gives a review of the clinical consequences of IRI in the context of liver surgery. Attention is drawn to the inflammatory mediators that are common to obesity in general and IRI. The relevance of IRI in the context of fatty liver becomes clearer in Chapter 3, where the post-laparoscopy inflammatory response is discussed. The chapter ends with a brief overview of the published literature on the effect of bariatric surgery and NAFLD/NASH.

2.2 NAFLD – a common entity

Although first described over 50 years ago, it is in the last 15 years that the clinical entity of non-alcoholic fatty liver disease (NAFLD) has been recognised as a significant problem **(122)**. Over 80% of patients undergoing bariatric surgery have fatty liver disease, compared with up to 15% of the non-obese population **(123)**. Non-alcoholic fatty liver disease (NAFLD) is characterised by hepatocyte accumulation of fat, with or without associated inflammation and fibrosis, in patients who do not consume more than 20 grams per day of alcohol or have viral hepatitis or metabolic storage disease, such as haemochromatosis. It is a spectrum of conditions extending from bland fatty steatosis, a relatively benign condition in itself, through non-alcoholic steatohepatis (NASH) and cirrhosis. Ludwig *et al* described NASH in 20 patients, commenting on the histological resemblance to alcoholic steatohepatitis and speculating about the significance of this pathology **(124)**. Up to 37% of obese individuals will have features of NASH **(123)**. Of these, a fifth may progress to liver cirrhosis.

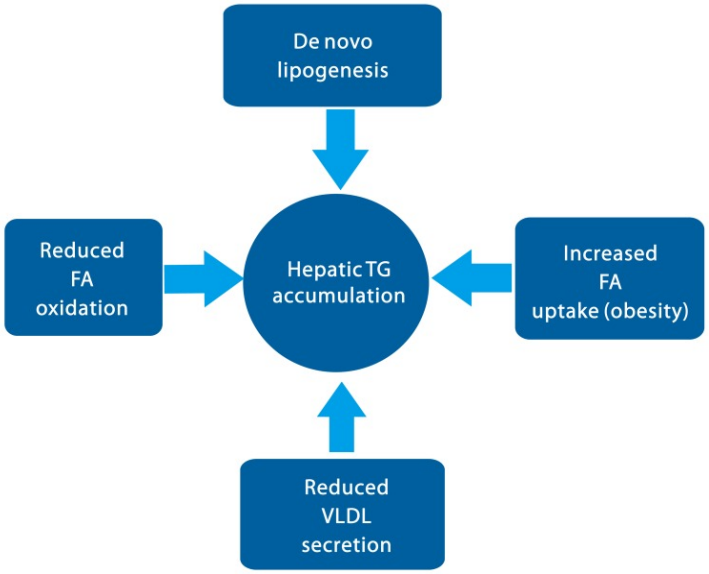


Figure 2.1 Factors leading to hepatic steatosis (reproduced from Koo *et al*, 2013) (125)

Changes in fatty acid (FA) metabolism occur following increased uptake and accumulation in the hepatocyte, stimulating triglyceride (TG) synthesis. Very low density lipoprotein (VLDL) secretion is concomitantly reduced, adding to the intracellular lipid load.

2.2.1 Pathophysiology of NAFLD

The accumulation of intracellular lipid, mostly triglyceride, is multi-factorial and thought to be due to changes in hepatocyte fatty acid metabolism, leading to increased uptake of free fatty acids, changes in oxidation of intracellular fats and reduced secretion of triglyceride, in the form of very low density lipoprotein (see Figure 2.1) **(125)**. Hepatic fatty acid metabolism is modulated by insulin and to a lesser extent other circulating hormones, including adiponectin. Impairment of mitochondrial fatty acid oxidation leads to production of fatty acid byproducts which themselves may exacerbate insulin resistance. Peroxisome proliferator-activated receptors (PPARs) and their co-receptor activators control the fatty acid oxidation and other important parts of energy metabolism **(126)**. The accumulation of intracellular lipid is thought to overwhelm the oxidative capacity of the cell and impair mitochondrial energy transfer. This causes depletion of adenosine triphosphate (ATP) and leads to increased production of reactive oxygen species (ROS), inducing oxidative stress within the cell. Berson *et al* showed that drugs such as amiodarone which are known to induce steatohepatitis in humans cause ATP depletion and lipid peroxidation (a marker of oxidative

stress) in rats **(127)**.

It is believed that such derangement of energy metabolism leaves fatty hepatocytes vulnerable to damage from toxins, ischaemia and infections. These insults constitute what is called the "second hit" **(128)**, leading to cytokine-mediated inflammation and eventually fibrosis **(129)**. These insults may trigger further liver injury, precipitating cirrhosis and even liver failure.

2.2.2 NAFLD – an important part of the metabolic syndrome

NAFLD is related to insulin resistance **(130)**. Systemic insulin resistance (IR) is associated with hyperglycaemia and hyperlipidaemia, contributing to the steatosis. Hepatic steatosis then leads to worsening hepatic IR, eventually leading to pancreatic endocrine failure and type 2 diabetes mellitus (T2DM) **(131)**. NAFLD and NASH comprise an important component of the metabolic syndrome (MetS), which can be defined as a combination of central obesity and any two of: hypertension, hyperglycaemia and dyslipidemia (see Table 1.1) **(33)**. NAFLD/NASH are significant predictors of cardiovascular disease, independent of diabetes **(122)**. NAFLD was associated with a higher pooled mortality rate compared with the general population (odds ratio, OR, 1.57, 95% Confidence Interval, CI, 1.18 – 2.10), with liver disease being the third most common cause of death compared with being the eleventh most common cause in the general population **(132)**. In a pooled analysis of studies determining the presence of NAFLD using either ultrasound or biopsy and controlling for presence of MetS, patients with NAFLD were at increased risk of cardiovascular disease (OR 1.75, 95%CI 1.38 to 2.23) **(132)**. The rate of survival of patients with simple steatosis was similar to the general population but patients with NASH had a higher pooled mortality (OR 1.81, 95%CI 1.24-2.66) **(132)**. The rate of progression of NASH to cirrhosis was between 13 and 25% depending on extent
of fibrosis in a Swedish study of 129 patients **(133)**. NASH has a profound impact on long term mortality. In NASH, liver-related mortality rate was 11-17% compared with only 1.7-2.7% in patients with simple steatosis (pooled OR 5.71, 95% 2.31 to 14.13) **(132)**.

Although ongoing excess intake of dietary calories is the most important factor in the exacerbation of IR and NAFLD, a number of pro-inflammatory hormonal changes occur. These changes create a chronic inflammatory state and drive the development of obesity-associated diseases, including atheromatous cardiovascular disease, as well as increase the chances of developing NASH (see Table 2.1). The increased deposition of central and peripheral adipose tissue not only exerts a mechanical effect, in terms of causing osteoarthritis, chronic back pain and obstructive sleep apnoea, but also is a major source of proinflammatory cytokines. These cytokines are thought to link obesity, NAFLD and IR **(134)**.

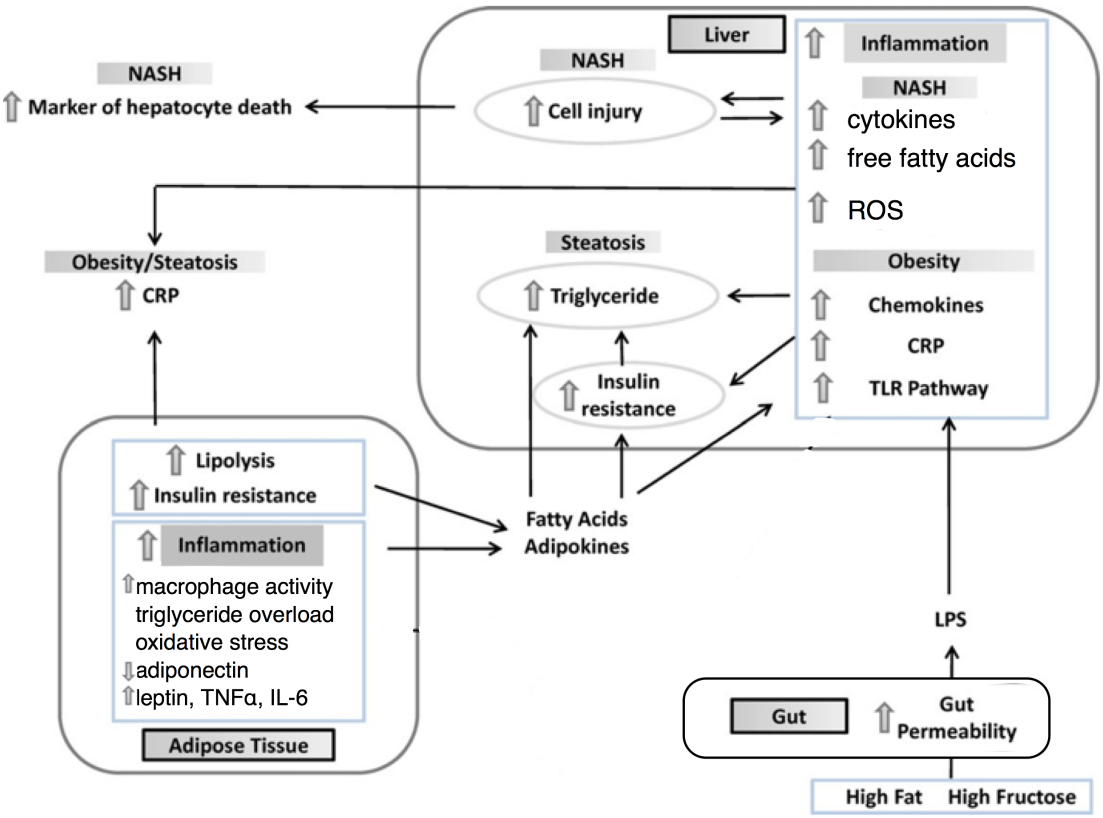


Figure 2.2 The link between steatosis, diet and inflammatory mediators in adipose tissue and liver, leading to hepatocyte injury (adapted from Tran and Gual, 2013) (135)

Pro-inflammatory cytokines and macrophage activation lead to production of ROS and cytokines in the vulnerable steatotic liver, where increased levels of FFA and activation of inflammatory and pro-apoptotic pathways leaves the liver vulnerable to further damage and development of NASH. Increased gut permeability and changes in

intestinal microbiota in obesity may exacerbate the inflammatory milieu, through absorption and circulation of lipopolysaccharides (LPS), toxic components of bacteria.

Table 2.1 Cytokines linking obesity, insulin resistance and NAFLD (adapted from O'Rourke *et al*, 2013) (134)

Mediator	Source	Physiological effects	Disease association
TNFα	• Macrophages • other immune cells • adipocytes	↑inflammation ↑diabetes	↑ in obesity ↑ metabolic syndrome
Leptin	• Adipocytes	↑inflammation ↑diabetes	↑ in obesity ↑ metabolic syndrome
IL-10	• Macrophages • other immune cells • adipocytes	↓inflammation ↑insulin release	↓in obesity ↓ metabolic syndrome
Adiponectin	• Adipocytes • Possibly liver	↓inflammation ↑insulin release	↓ in obesity ↓ metabolic disease
IL-6	•Macrophages •other immune cells •adipocytes	↑inflammation ↑diabetes	↑ in obesity ↑ metabolic syndrome
IL-1	• Macrophages • other immune cells	↑inflammation ↑diabetes	↑ in obesity ↑ metabolic syndrome
IFN-γ	• T cells • NK cells	↑inflammation ↑diabetes	Unknown effect on DM possibly ↑ in obesity
Resistin	• Macrophages • adipocytes	↑inflammation ↑diabetes	↑ in obesity ↑ metabolic syndrome
CCL2	• adipocytes	↑inflammation ↑diabetes	Unknown effect diabetes Likely ↑ in obesity

2.3 Development of NASH

2.3.1 Role of inflammation and oxidative stress

Within the liver, the change in the cytokine milieu and the reduction in the capacity of the fat-laden hepatocytes to tolerate any injury lead to increased levels of hepatocellular injury and death **(135)**. Figure 2.2 depicts this sequence of events leading to hepatocyte injury and NASH. Crespo *et al* measured TNFα mRNA expression in liver and peripheral adipose tissue in 52 obese patients and showed that TNFα mRNA is overexpressed in patients with NASH **(136)**. Overexpression

increased with extent of fibrosis and severity of NASH. Similarly, Wieckowska *et al* found that both circulating plasma IL-6 and hepatic IL-6 mRNA expression is increased in patients with NASH and levels correlate with the extent of fibrosis **(137)**. The increased expression of these pro-inflammatory mediators is associated with increases in the expression of other genes involved in upregulation of the inflammatory response, recruitment of immune cells and activation of the toll-like receptor pathways (TLR) **(138)**. TLR are thought to play an important role in progression of simple steatosis to NASH and may also be activated by changes in gut microbes and other environmental pathogens **(139)**. The chronic imbalance within hepatocytes towards a state of oxidative stress leads to activation of hepatic NF-κβ and activating protein-1 (AP-1), through activation of the TLR pathway. Videla *et al* demonstrated increased expression of NF-κβ and AP-1 in NASH livers compared with bland steatotic livers, which correlated with increased markers of oxidative stress and insulin resistance **(140)**.

Pro-inflammatory cytokines, including TNFα, are associated with increased levels of intracellular oxidative stress **(141)**. This may be exacerbated by insulin resistance, which is known to cause induction of mitochondrial enzymes within the liver, including the cytochrome-P450 enzymes involved in oxidation of lipids, steroids and other organic substrates, including drug metabolism **(142)**. Upregulation of these enzymes has been demonstrated in both cell and animal models of NASH, and are associated with increased free radical production **(141)**. Increased markers of lipid peroxidation, including malondialdehyde (MDA) and TBARS, are associated with NASH and steatosis **(143)**. Lipid peroxidation is also detected systemically and correlates with the presence of NASH, compared with simple steatosis **(144)**. The development of fibrosis is also related to tissue levels of MDA **(145)**. The processes are summarised in Figure 2.3.

2.3.2 Apoptosis and NASH

The final common effect of these inflammatory stimuli in progression of NAFLD to NASH is the initiation of hepatocyte apoptosis. Apoptosis (programmed cell death) can occur via two main pathways – the extrinsic pathway is that which is initiated

by ligand binding and activation of a plasma membrane "death" receptor, such as the tumour necrosis family of receptors; and the intrinsic pathway, which originates from the mitochondria, and involves a loss of one of many activities that continually suppress apoptosis **(146)**. Thus, some cytokines such as Fas Ligand (FasL) and tumour necrosis factor-related apoptosis inducing ligand (TRAIL) may induce apoptosis via the extrinsic pathway, whilst intracellular changes such as oxidative stress and lipid peroxidation may lead to activation of the intrinsic pathway. The detection of higher levels of products of apoptosis, such as cytokeratin-18 (CK-18) fragments, in patients with NASH and fibrosis along with overexpression of death receptors such as Fas and the TNFα receptor in NASH liver tissue have highlighted the importance of apoptosis in NASH/NAFLD **(135)**.

CK-18 is a major intermediate filament protein in the liver, which form an cytoskeleton within the cell, associating with the plasma membrane forming a scaffold within the cell **(147)**. CK-18 is cleaved by caspase enzymes during apoptosis and cell death and can be detected in the plasma. Two commercially available CK-18 assays are able to detect different epitopes of CK-18 M65 detects the full length protein released during cell degradation and provides a measure of total cell death, whilst M30 detects a shorter fragment of CK-18 which is only found after caspase-mediated cleavage, acting as a measure of apoptotic cell death. Wieckowska, Feldstein and colleagues established that CK-18-M30 levels were increased in NASH compared with obese NAFLD controls and correlated with fibrosis scores **(148, 149)**. Similar relationships between CK-18 fragments and disease severity and liver fibrosis have been established in viral and alcoholic hepatitis **(150)**. In a mouse model of NASH, mice fed with a pan-caspase inhibitor for 4-8 weeks showed decreased histological evidence of liver fibrosis as well as reduced hepatic collagen mRNA expression **(151)**. The pathway through which apoptosis causes fibrosis is not fully elucidated, and involves hepatic stellate cells (HSC, see Figure 2.3). These reside in the perisinusoidal tissue and act as antigen-presenting cells. It is thought that the remnants of apoptotic cells stimulate HSC activity, leading to the deposition of collagen **(152)**. A model of pulmonary fibrosis has shown that there may be a bidirectional relationship with activated HSC stimulating Fas-mediated apoptosis as well **(153)** cited in **(152)**.

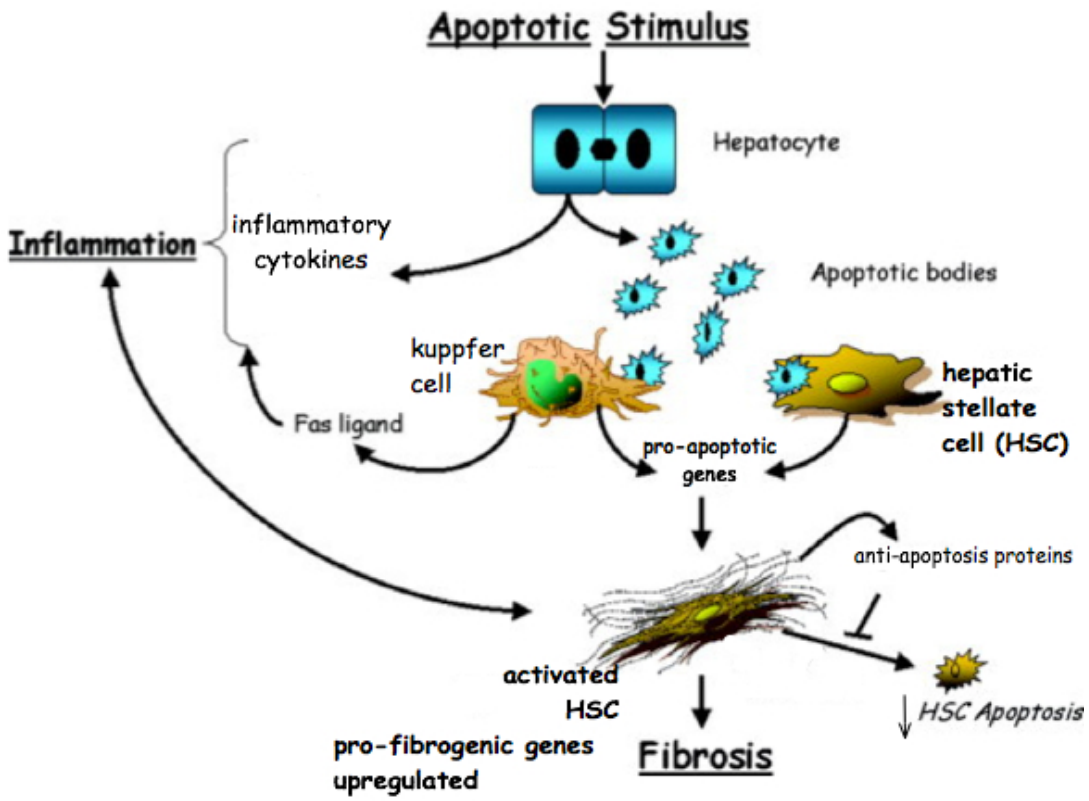


Figure 2.3 Inflammation and Apoptosis lead to fibrosis and inflammation(adapted from Canbay *et al*, 2004) (152)

Following an apoptotic stimulus, including pro-inflammatory cytokines like TNFα and cellular injury secondary to ischaemia, hepatocyte apoptosis occurs. Signals to active immune cells resident in the hepatic parenchyma, including macrophages, Kupffer cells and hepatic stellate cells (HSC) leads to uptake of cellular remnants of apoptosis (apoptotic bodies). This in turn stimulates release of death ligands (FasL) and expression of pro-fibrogenic genes, leading to fibrosis and further inflammation. Gene expression of anti-apoptosis pathways within activated HSCs is also increased, so that these cells may remain active. The whole process is another vicious cycle. Previously apoptosis was thought to be a "neutral" event that did not produce further inflammatory stimuli.

2.3.3 Other pathological mechanisms of progression of NAFLD to NASH

Much of the newer evidence has come from cross-sectional studies of bariatric surgery patients. Vitamin A is an important non-enzymatic cellular antioxidant and NAFLD is associated with a decrease in its circulating levels (as retinol), reflecting depletion of antioxidant reserves over time, increasing the risk of progression to steatohepatitis and fibrosis. In a study of paired liver and serum

samples, Chaves *et al* found that increasing severity of NAFLD was associated with deficiency of serum and liver retinol concentrations **(154)**. NAS correlated with liver retinol concentration significantly, suggesting that local depletion is more important in the progression of NAFLD. Folate deficiency is also associated with NAFLD and may amplify oxidative stress **(155)**. Liver tissue concentrations of hydroperoxides, a tissue marker of oxidative stress, are increased in NASH compared with simple steatosis. **(156)**

Aberrant Cytochrome P450 overactivity is thought to cause lipid peroxidation and hepatocyte injury, with particular interest in *CYP2E1* which is overexpressed in human studies and animal models. Using immunohistochemistry to stain tissue with anti-*CYP2E1* and anti-MDA antibodies, Bell *et al* found that liver biopsies after bariatric surgery had decreased levels of lipid peroxidation and CYP2E1 activity **(143)**. Decrease in steatosis correlated significantly with the decrease in MDA and *CYP2E1* staining.

Although unregulated free fatty oxidation (due to excess intracellular lipid) is an important step in the production of ROS, some fatty acids may have a protective effect **(157)**. Depletion of liver n-3 long-chain polyunsaturated fatty acid (LCPUFA) is associated with oxidative stress and insulin resistance. Enzymatic desaturation of PUFAs plays an important role in maintaining physiological balance of lipogenesis and free fatty acid oxidation. In obese NAFLD patients, desaturase activity is decreased along with levels of LCPUFA, and these decreases correlate with oxidative stress and insulin resistance **(158)**. Along with high levels of glucose and insulin that stimulate fatty acid synthesis, these changes may be an important mechanism by which fat accumulation in the liver occurs **(140)**. After surgery, total fat mass, including volume of visceral and subcutaneous mass, decreases significantly, which correlate with decreased plasma concentration of derivatives of reactive oxidative metabolites, another measure of oxidative stress **(159)**.

2.4 Diagnosis of NASH

2.4.1 Liver Biopsy

There is no consistent relationship between serum liver function tests and extent of fatty infiltration, fibrosis and inflammation. Diagnosis of NAFLD is made histopathologically, with liver biopsy. Liver biopsy carries a quoted 1:300 risk of major haemorrhage **(160)**, although image guidance in practice may make it safer **(161)**. The severity of liver damage ranges from simple steatosis (bland fatty infiltration) to inflammation (steatohepatitis, NASH), fibrosis and liver cirrhosis. Brunt and colleagues have standardised the histopathological criteria for NAFLD/NASH. They conducted a large scale multi-centre study, employing a long list of standardised histological variables. Using logistic regression, they identified the variables most sensitive in making a diagnosis of NASH **(162)**. These scored variables were measurements of the extent of steatosis, hepatocyte ballooning and lobular inflammation and comprised the NAFLD Activity Score (NAS), with a NAS≥5 defining the threshold for NASH (see Table 2.2). The same group of authors highlighted the limitations of this semi-quantitative score when they reviewed the scores and individual histology of 976 patients included in their clinical research network's studies. They found that only 75% of patients with definite histological evidence of steatohepatitis had a NAS≥5, with 29% of patients with NAS≤4 having definite evidence of steatohepatitis **(163)**. Most importantly, they concluded that the diagnosis of NASH was strongly associated with clinical features of MetS. There is also considerable variability of histological findings in biopsies from an individual, depending on location and technique. Maharaj *et al* found approximately 50% of 75 patients had diagnostic agreement between three consecutive needle biopsies from the same entry site **(164)**. Merriman *et al* found only fair to moderate agreement in the assessment of fibrosis, necrosis and inflammation and between 41 paired liver biopsies taken from right and left lobes of the liver **(165)**. Similarly Larson *et al* found only 74% agreement in assessment of inflammation in 43 paired liver biopsies, although agreement for overall NAS≥5 was 93% **(166)**. Padoin *et al* concluded that extent of fibrosis might be overestimated by wedge liver biopsy when compared with needle biopsy, perhaps due to the increased amount of collagen in the periphery of the liver **(167)**. Thus,

although liver biopsy remains the gold standard, it does have flaws and appreciable risks.

Table 2.2 Components of NAFLD Activity Score and Brunt-Kleiner Fibrosis Staging (162)

NAS Score	**0**	**1**	**2**	**3**	
Steatosis	<5%	5–33%	34–66%	>66%	
Ballooning	None	Rare or few	Many		
Lobular Inflammation (per x200 field)	None	1–2 foci	2–4 foci	>4 foci	
Fibrosis Score	**F0**	**F1**	**F2**	**F3**	**F4**
Fibrosis	No fibrosis	Zone 3 peri-sinusoidal fibrosis only	Zone 3 plus portal/ periportal fibrosis	As above with bridging fibrosis	Cirrhosis

Note that these components should be assessed by an experienced histopathologist. NAS ≥5 is predictive of a diagnosis of NASH but this cutoff score cannot be used as the sole criterion for diagnosis of NASH. Judging the presence of NASH (versus simple steatosis) can only be made after an expert assessment to look for the pathognomonic hallmarks of steatohepatitis.

Given that simple steatosis has a relatively benign course compared with NASH **(132)**, most efforts to improve non-invasive diagnosis are aimed at detecting steatohepatitis and/or fibrosis. The aim is to stratify patients into high and low-risk pools, with definitive liver biopsy reserved for patients at high risk of NASH with or without fibrosis.

2.4.2 Non-invasive diagnosis of NAFLD and NASH

The detection of steatosis itself is relatively straightforward **(162)**. Given its high prevalence in the obese population, even a combination of simple routine parameters may be used to accurately predict steatosis. The fatty liver index is calculated using body mass index (BMI), waist circumference and gamma-GT, with

an accuracy of 84% **(168)**. Transabdominal ultrasound scanning has a sensitivity of 60-94% and specificity of 66-95% in detecting fatty liver, with much higher accuracy in detecting moderate or severe steatosis **(169)**.

Of more clinical relevance are the techniques to detect fibrosis and NASH. Transient elastography (TE) is an ultrasound-based method of measuring the stiffness of liver tissue, a correlate of fibrosis. TE had a sensitivity in six studies had a pooled sensitivity of 79% in detecting Brunt-Kleiner score F2 fibrosis and 94% in detecting F3 fibrosis **(132)**. However, mean BMI for patients in these studies is 30kg/m^2, indicating that the ability of TE to record a measurement is impaired by morbid obesity. Other imaging-based methods of fibrosis assessment include computed tomography (CT) and magnetic resonance (MR) imaging. Both are relatively expensive and, in the case of CT, associated with risks of radiation exposure **(169)**. Accuracy in detecting and grading fibrosis is still relatively poor and at present not used in routine practice. In a recent study of 113 patients undergoing contrast enhanced MRI to detect fibrosis, Jang *et al* report a sensitivity of only 50% for F2 fibrosis, and 83% for F4 fibrosis, although respective specificities were >90% **(170)**. MR elastography is a more promising technique, with a pooled sensitivity of 92% (95% CI 85-96%) and specificity of 96% (95%CI 91-98%) in distinguishing F1/F2 fibrosis from F3/F4 in a meta-analysis of 5 studies **(171)**.

2.4.3 Non-invasive diagnosis of fibrosis

There has been great interest in circulating biomarkers of fibrosis and NASH. For NASH and fibrosis, a number of other clinical and biochemical measures have been combined to give a predictive score, including demographic details and specific biomarkers. Table 2.3 gives examples of these. Sensitivities and specificities are in the region of 80-90% but unfortunately none of these models have been externally validated on large cohorts **(172)**. Some of these scores include hyaluronic acid (HA). This is an extracellular matrix protein found in connective tissue and involved in cell proliferation and migration. In alcoholic liver disease, it is used as a marker of cirrhosis and fibrosis with high sensitivity and moderate specificity **(173)**. In the setting of NAFLD/NASH, it has been used in combination with other markers (for example in the Enhanced Liver Fibrosis panel **(174)**) and clinical parameters **(175)**.

Table 2.3 Selection of Predictive Scores for diagnosis of NASH and Fibrosis (based on tables in Castera *et al*, 2013 (175) and Musso *et al*, 2011 (132))

Predictive Score	Patients n=	Parameters	Endpoint	Cut-offs	AUROC	Se (%)	Sp (%)	PPV	NPV
Nash Test	257 NAFLD 383 control group	Age, gender, BMI, triglycerides, cholesterol, α-2-macroglobulin, GGT, AST, ALT, haptoglobinapolipoprotein A1, total bilirubin	NAS ≥ 5 (Kleiner)	ND	0.79	29	98	91	71
Palekar index for NASH	80 NAFLD (39 SS* and 41 NASH)	Age ≥ 50 yrs, female gender, AST ≥ 45 U/L, AST/ALT ratio ≥ 0.8, BMI ≥ 30 Kg/m^2, hyaluronate ≥ 55 mcg/l	NASH (Brunt)	≥3	0.76	74	66	68	71
CK-18 for NASH	139 NAFLD 150 health controls	Serum fragment of cytokeratin-18	NAS ≥ 5 (Kleiner)	>246 >279	0.83	75 71	81 85	ND	ND
NASH Clinical score for morbid obesity	200 obese	hypertension, diabetes, AST ≥ 27 IU/L, ALT ≥ 27 IU/L, obstructive sleep apnea and nonblack race	NAS ≥ 5 (Kleiner)	≥6	0.80	22	99	80%	93%
NAFLD Fibrosis Score	733	-1.675 + 0.037 × age (years) + 0.094 × BMI (kg/m2) + 1.13 × IFG/diabetes (yes = 1, no = 0) + 0.99 × AST/ALT ratio – 0.013 × platelet (×109/l) – 0.66 × albumin (g/dl)	F0-2 or F3/4 (Brunt-Kleiner Fibrosis staging)	< -1.455 = F0-F2 > 0.675 = F3-F4	0.88	F0-2 82 F3/4 51	F0-2 77 F3/4 98	56 90	93 85
Enhanced Liver Fibrosis Panel	196	ELF Panel: Hyaluronic Acid, P3NP, TIMP1, calculated using a proprietary algorithm	F0/1 or F2-4 (Brunt-Kleiner Fibrosis Staging)	–0.3625	0.90	80	67	62	84

AUROC -area under ROC curve, Se – sensitivity, Sp – specificity, PPV - positive predictive value, NPV - negative predictive value, AST - aspartate aminotransferase, ALT -alanine aminotransferase, GGT - gamma glutamyltranspeptidase, BMI - body mass index, NAS - NAFLD activity score, ND – not described.

2.5 Ischaemia-reperfusion injury and fatty liver

2.5.1 Ischaemia-reperfusion injury

Disruption of the blood supply to the solid organs, either through direct pressure on the organ itself or by occlusion of the arterial blood flow, causes ischaemia. This ischaemia may occur deliberately, such as part of a surgical procedure where the arterial supply is clamped, or inadvertently as a secondary effect of another action, such as creation of a surgical pneumoperitoneum or application of a retractor (as discussed in this thesis, see Chapter 3). Ischaemia leads to depletion in intracellular oxygen and adenosine triphosphate (ATP), the basic currency of cellular energy transfer **(176)**. The resulting metabolic changes, including a switch to glycogen consumption and anaerobic respiration, leads to disruption of the cell membrane and intracellular oedema, sodium accumulation and derangement of intracellular calcium, with production of reactive oxygen species (ROS) and inflammatory cytokines **(177)**.

Within the liver, a type of macrophage called Kupffer cells line the sinusoids **(178)**. These immune cells are part of the mononuclear phagocyte lineage. As well as scavenging red blood cells, they are involved in the response to gut-derived microbes and circulating toxins. Ischaemia leads to aberrant Kupffer cell activation, with production of cytokines, vasoactive compounds and chemoattractants, in turn producing a neutrophil response and platelet aggregation. This in turns triggers activation of both the humoral and non-humoral immune response, including complement activation (as depicted in Figure 2.4) **(179)**.

Continued ischaemia would of course lead to death, at cellular, organ and subsequently organism-level. However, non-lethal transient ischaemia is followed by reperfusion of the organ, usually upon release of the obstruction to the blood supply. In the liver, restoration of the blood supply leads to exacerbation of the above effects. Increases in pro-inflammatory cytokines, damage to the sinusoidal endothelium and increased expression of cellular adhesion molecules all lead to neutrophil and platelet aggregation. In some smaller capillaries, blood flow may be completely occluded, worsening the ischaemic damage to the cells

locally **(179)**. Aggregation of neutrophils leads to increased migration of activated neutrophils into the liver parenchyma, increasing the production of cytokines and reactive oxygen species. This aberrant activation of the immune response is a vicious cycle, causing damage to hepatocytes and sinusoidal endothelial cells, manifested as derangements in liver function, systemic inflammatory response and even organ failure **(177)**.

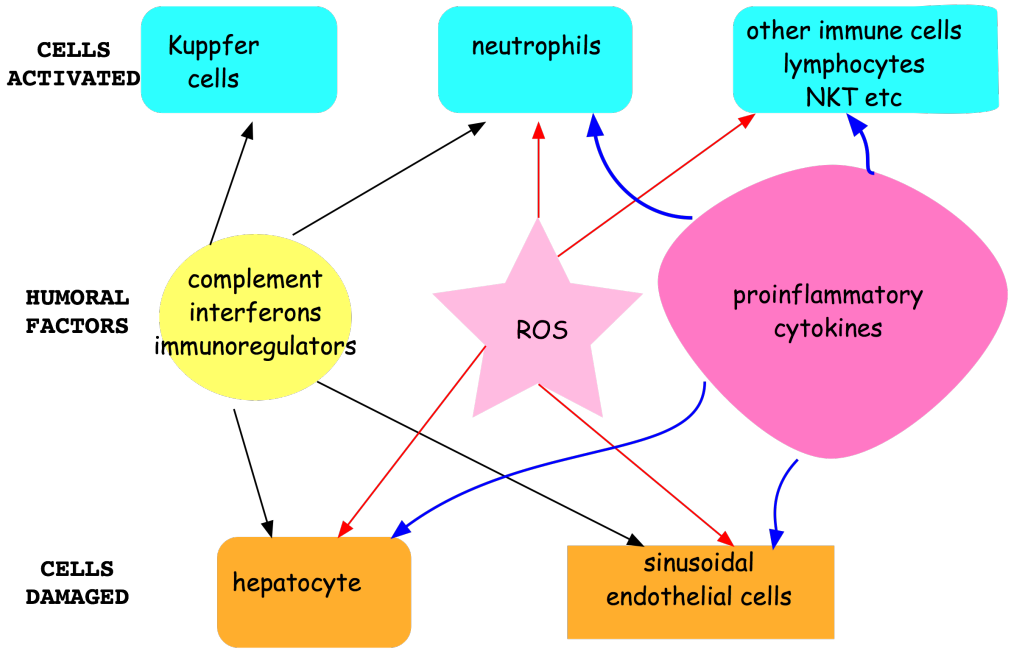


Figure 2.4 The interplay of cellular and humoral factors in liver ischaemia-reperfusion injury (adapted from Abu-Amara *et al*, 2010) (177)

Humoral factors, including complement, reactive oxygen species (ROS) and proinflammatory cytokines, lead to activation of immune cells as well as direct toxic damage to hepatocytes and sinusoidal endothelial cells (SECs). This diagram is a simplification because the interplay is not as linear as depicted. Activated immune cells also produce many of the humoral factors themselves and go on to damage hepatocytes and SECs. NKT – natural killer T cells

The main mediators of ischaemia-reperfusion injury (IRI) within the liver are ROS, cytokines and complement. ROS are produced during aerobic metabolism, and are neutralised by various cellular antioxidant defense mechanisms. In IRI, the antioxidant capacity may be overwhelmed, leading to oxidative stress and further cellular damage. Cytokines implicated in IRI include interleukins (IL) 1 and 6 and tumour necrosis factor (TNF) α. Complement activation leads to direct injury to the liver by deposition of membrane attack complexes, the final element in the

complement pathway, and also stimulates further cytokine release and production of neutrophil chemoattractants (process summarised in Figure 2.5).

Hepatocyte damage may have various consequences, depending on severity. Non-lethal injury may lead to changes in gene expression and intracellular metabolism in a way that may obviate the damage and allow the cell to repair itself. Cytokine activation, especially TNFα, may trigger apoptosis and cell death. A more damaging mode of cell death is through necrosis, which is an uncontrolled process leading to a potential release of more proinflammatory stimuli and toxins. The extent of necrosis will depend not only on the severity of the initial insult but also the underlying condition of the liver, with steatosis and other chronic underlying conditions associated with increased necrosis and worse outcomes.

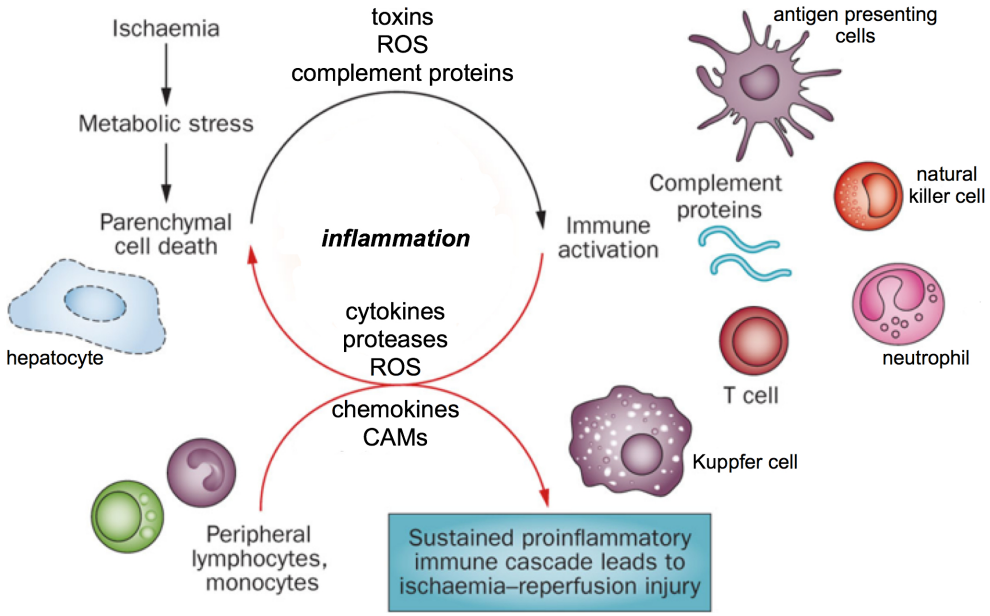


Figure 2.5 Summary of the processes leading to ischaemia-reperfusion injury in the liver (taken and adapted from Zhai *et al*, 2013) (180)

Reduction in hepatic parenchymal perfusion (local or global) results to reduced tissue oxygen availability and depletion of adenosine triphosphate, the basic unit of cellular energy transfer. This leads to an ischaemic injury and cellular death, causing a release of cellular proteins. These are immunologically active and potentially toxic to the local environment. Reactive oxygen species (ROS) are produced after restoration of the blood supply and re-oxygenation, by which time mitochondrial metabolism is disturbed. The combination of ROS and cellular proteins lead to activation of immune cells within the parenchyma, including Kupffer cells, T cells, Natural Killer cells, antigen presenting (dendritic) cells and neutrophils. Further recruitment of other immune cells from the circulation by increased expression of chemotactic signals and adhesion molecules sustains the pro-inflammatory cascade, worsening the extent of parenchymal damage.

2.5.2 Fatty livers are more vulnerable to injury

Fatty liver has a number of structural and metabolic differences to non-steatotic liver **(181)**. As a consequence, tolerance to ischaemia is reduced. In a study of lean and obese rats subjected to hepatic ischaemia and reperfusion, Selzner *et al* demonstrated that obese rats were unable to survive an experimental insult of 60 minutes of total hepatic ischaemia, whereas all lean animals survived. Furthermore, they demonstrated that the number of apoptotic cells in the liver rise with increasing periods of ischaemia, but the relative increase in apoptosis is much smaller in obese rats, with a larger amount of necrosis, when compared to lean rats. Post-IRI apoptosis is mediated by the caspase cascade. Selzner *et al* found that administration of a caspase inhibitor reduced AST levels in lean rats but had no effect on obese rats. Taking their findings together, they were able to conclude that the mode of cellular damage and death differs from non-steatotic liver, with a greater degree of necrosis rather than apoptosis in steatotic livers **(182)**. In a comparative study using wild-type rats and a rat model with hepatic steatosis, evaluating the effect of administration of methylprednisolone prior to induction of liver ischaemia by clamping the inflow, Saidi *et al* demonstrated reduced inflammatory response and histological evidence of IRI in treated wild-type rats compared with controls. The beneficial effect of steroids was not seen in the steatotic rats, indicating that different mechanisms governing the inflammatory response and cellular damage/death were present in lean and steatotic livers **(183)**.

Investigation of the effects of steatosis on hepatocyte function has been extensive and revealed a number of metabolic and structural changes that leave fatty liver vulnerable to ischaemic insults. Intracellular accumulation of lipid leads to displacement of the nucleus to the edge of the cell and ballooning. Seifalian *et al* used Doppler flowmetry and intravital microscopy to demonstrate that parenchymal perfusion is reduced in fatty livers compared with healthy, both in human donor livers and a rabbit model **(184)**. Reduced flow is also seen in fibrotic rat livers and can be improved mechanically with increased portal perfusion pressures **(185)**. These changes lead to narrowing of the sinusoidal capillaries and the sluggish flow leads to trapping of white blood cells, with concomitant

activation of immunologically active Kuppfer cells, stellate cells and macrophages (summarised in Figure 2.6) **(186)**.

In general, steatotic hepatocytes are subjected to a chronic inflammatory stimulus, leading to changes in energy homeostasis. Impairment of mitochondrial energy generation, via the production of ATP, means that during reperfusion after a period of ischaemia, the switch from anaerobic metabolism back to oxidative phosphorylation is impaired **(181)**. Thus, fatty hepatocytes are unable to recover following ischaemia and are more likely to die, more readily through necrosis than apoptosis.

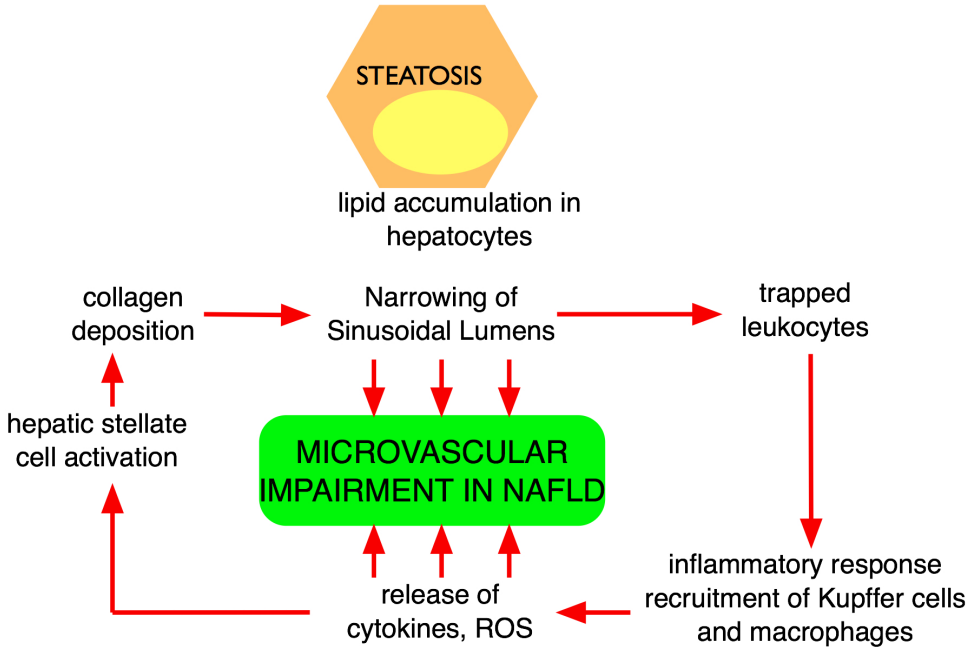


Figure 2.6 Factors leading to microcirculatory impairment associated with fatty liver disease (adapted from Farrell *et al*, 2008) (186)
ROS – reactive oxygen species

2.5.3 Cellular mechanisms of injury in steatotic livers

An analysis of all of the exact cellular mechanisms of this process are beyond the scope of this review. The main mechanisms are discussed here and a fuller review is provided by Tashiro *et al* **(181)**. Much interest is centred on the role of mitochondrial uncoupling protein-2 (UCP-2). UCP is usually only found in Kuppfer cells and is involved in the uncoupling of oxidation of energy substrates from the production of ATP **(187)**. In fatty liver, UCP-2 is expressed in hepatocytes as well as Kuppfer cells **(188)**. In an obese mouse model, Fulop *et al* demonstrated that

Fas-induced liver injury in UCP-2 deficient mice is reduced compared with UCP-2 competent animals **(189)**. When compared with the UCP-2 competent animals, liver ATP level in the UCP-2 deficient mice was significantly reduced. Malondialdehyde (MDA) levels were used as a measure of oxidative stress and MDA was not different between groups, indicating that oxidative stress was not a major factor in causing Fas-mediated liver necrosis. Interestingly, UCP-2 expression was decreased in Kupffer cells and the authors speculate that its relative absence lead to increased production of reactive oxygen species from Kupffer cells. The inference is that normal UCP-2 expression in Kupffer cells means that, in conditions of increased oxidative stress, Kupffer cell ATP metabolism is reduced by the uncoupling action of UCP-2, which is a protective mechanism **(187)**. In hepatocytes, UCP-2 has the opposite effect, and its abundant expression in fatty liver has a pathological effect, meaning that the cells are more vulnerable to liver injury **(181)**.

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear hormone receptors that are implicated in the pathogenesis of NAFLD and the impairment of the response to IRI. The balance of two of the main isoforms, PPARα and PPARγ, is reversed in fatty liver **(126)**. PPARα is involved in the inflammatory response. Teoh *et al* showed that administration of a PPARα agonist in mice with steatosis leads to reduction in liver lipid content. Furthermore, in their IRI model, pre-treatment with PPARα agonist was associated with reduced hepatocyte necrosis, which correlated with reduced levels of inflammatory cytokines including TNFα and IL-6 and reduced levels of adhesion molecules **(190)**. These changes were associated with increased NF-κβ activation. As discussed above, NF-κβ plays a complex role both in mediating inflammatory response to IRI and the recovery phase **(191)**. Massip-Salcedo *et al* showed that the effect of an PPARα agonist on a rat model of IRI is similar to the effect of preconditioning, with the effect of preconditioning negated by administration of a PPARα antagonist **(192)**.

2.5.4 Adiponectin –linking between obesity, steatosis and liver injury

Adiponectin may also play an important role in IRI in steatotic livers. Circulating adiponectin is reduced in obese subjects, which is thought to have a deleterious effect on health, as adiponectin has anti-inflammatory effects **(193)**. Conversely adiponectin is found in higher levels in steatotic liver compared with healthy liver **(192)**. Massip-Salcedo *et al* found that adiponectin mRNA expression increased more in steatotic livers after IRI compared with normal liver controls. Confusingly, they also showed that by blocking the effect of adiponectin with an inhibitor RNA, IRI-induced hepatocellular damage and oxidative stress was reduced in steatotic livers but not in normal livers **(192)**. Man *et al* showed that addition of exogenous adiponectin in a rat liver transplantation model improved outcomes in otherwise small-for-size transplants **(194)**. They concluded that adiponectin's anti-inflammatory effects may reduce IRI-induced injury and may also exert an anti-obesity effect by clearing intracellular lipid, improving the function of the graft. Adiponectin is now known to mediate several intracellular processes involved in glucose metabolism, insulin sensitivity, lipid clearance, nitric oxide production and cytokine expression, as well as exerting an effect on cellular energy metabolism by increasing fatty acid oxidation **(193)**. Although its exact role in IRI is unclear, it is apparent that obesity and steatosis are closely linked with circulating and intrahepatic adiponectin expression **(195)**. The same authors have recently shown in a rat model that transplanted steatotic livers downregulate adiponectin and resistin production and that increasing their levels in the graft reduces evidence of hepatocellular injury after transplantation **(196)**.

2.6 Clinical Consequences of IRI

2.6.1 IRI in organ transplantation

The majority of both clinical and laboratory investigation of IRI is in the field of liver and kidney transplantation. The process of organ retrieval from the donor, transport to the recipient and then implantation leads to prolonged times of warm (that is, still at or near body temperature whilst without a blood supply) and cold ischaemia. Whilst still warm, the metabolic processes triggered by ischaemia persist and it is imperative to cool the organ using infused fluids as fast as possible and remove it from the body to a cold environment. Reperfusion after implantation leads to IRI. Primary non-functioning of liver grafts is approximately 6 % (range 0.6-22%) with worse outcomes for grafts that are fatty or have long warm ischaemia times **(197)**. Primary non-function requires re-transplantation whereas initial poor functioning of grafts is more common, occurring in 15-30% and associated with increased rates of systemic complications, for more interventions and longer hospital stays **(197)**. Similarly, acute kidney injury occurs in up to 20% **(198)** of patients and is associated with prolonged warm ischaemia times, amongst other factors **(199)**. Patients with acute kidney injury have a higher risk (hazard ratio 2.74) of graft failure compared to patients without **(200)**.

In response to this problem, there have been multiple trials of pharmacological agents targeted at reducing or preventing IRI (see Table 2.4 **(180)** for examples in liver transplantation). These have included interventions targeted at the donor (pre-retrieval), at the graft itself via the transplant preservation solution and at the recipient patient. Although a number of studies showed improvements in primary laboratory outcome measures, all the trials have been too small to show a definite improvement in overall outcomes for the intervention group.

Table 2.4 Randomised studies using various pharmacological interventions to minimise IRI in liver transplantation using deceased donors (adapted from a review by Zhai *et al*, 2013){Zhai, 2013 #6}

Study	Pharmacological intervention	Mechanism	Patients (placebo/ study drug)	Findings for intervention group
Klein *et al* (1999) **(200)**	Epoprostenol (iv bolus of 500 µg) before cross clamp	Improvement of sinusoidal perfusion	53/53	Decreased levels of AST and ALT after surgery
Bogetti *et al* (2005) **(201)**	Thymoglobulin during anhepatic phase and 2 doses after surgery	Suppression of inflammatory immune response	11/11	Decreased levels of AST and bilirubin after surgery, improved initial allograft function
Khan *et al* (2005) **(202)**	N-acetylcysteine (NAC) iv and portal flush of donor liver	Antioxidant hepatoprotection	9/9	No protective effects on liver IRI or on acute cellular rejection
Baskin-Bey *et al* (2007) **(203)**	IDN-6556 in organ storage solution and recipient	Inhibition of pan-caspase (apoptosis)	23 (placebo)/23/ 27/26	Decreased apoptosis and decreased liver injury for the group with study drug in preservation and flush solution
Lang et a (2007) **(204)**	Inhaled nitric oxide (NO, 80ppm) during liver transplantation	Downregulation of endogenous NO production	10/10	Decreased levels of AST after surgery, decreased hepatocyte apoptosis, improved rate of liver function
Kotsch *et al* (2008) **(205)**	Donor treatment with iv methylprednisolone before recovery	Suppression of inflammatory immune response	50/50	Decreased levels of AST, decreased serum levels of cytokine, improved levels of biomarkers after surgery and decreased incidence of acute rejection
Hilmi *et al* (2010) **(206)**	N-acetylcysteine (12 doses) in liver transplant patients	Antioxidant/glutathione-mediated hepatoprotection	50/50	No effects on liver/renal injury, no increase in GSH levels in some patients (possibility of inadequate dose/duration of NAC)

2.6.2 Ischaemia-Reperfusion Injury in hepatic resection

In a non-transplant setting, the discrete role that IRI plays in post-operative complications is more difficult to discern. Although there is a rise in inflammatory markers following liver surgery, the rise is similar to that after other major abdominal surgery and is not correlated with deranged liver function **(201)**. IL-6 and IL-10 increase after major liver surgery and correlate with operating time and are predictors of post-operative complications and liver dysfunction **(202)**.

Clinical pre-operative predictors of complications and increased inflammatory response are the same factors associated with IRI **(203)**. Thus, markers of inflammatory response (including cytokines, CRP) may be taken as surrogate indicators of IRI in this group. van de Poll *et al* measured markers of liver injury and inflammation after liver resection with and without occlusion of the liver inflow blood supply (Pringle manoeuvre) and showed that liver injury occurs on manipulation of the liver and inflow occlusion does not have a significant additive effect on liver injury **(204)**. This study does not account for the inherent delays in cytokine expression and no comparisons in post-operative period are given. Nonetheless, IRI and the measured inflammatory response are not the only major predictor of complications, as other patient and technical factors, such as pre-existing cardiovascular disease, long operating times, high blood loss and development of complications like bile leak, are more important **(205)**.

Despite these limitations, there has been much interest in manipulation of the inflammatory response **(206)** in an attempt to improve outcomes. Steroid administration has been the most effective method of reducing IRI. A meta-analysis of six studies including 396 patients showed that steroid administration was associated with lower post-operative day 1 IL-6 and day 3 CRP, with a significantly lower rate of morbidity in the steroid group (relative risk 0.76, 95% confidence interval 0.57 to 0.99), p=0.047) **(207)**. Interestingly, pooled analysis of ALT levels were similar in steroid and control groups.

Ischaemic preconditioning (IPC) is another attempt to ameliorate the effects of IRI by subjecting the liver to a short period of ischaemia and reperfusion before the

main operative insult occurs. A number of animal models and varying techniques have been shown to produce a favourable response. The underlying mechanism is questioned, but is thought to involve an increase in nitric oxide **(208)**, associated with increased tolerance to the effects of oxygen depletion within the mitochondria with preservation of ATP metabolism **(209)**. Within the field of liver resection, a short period of inflow occlusion is performed without liver resection in an effort to precondition the liver for longer periods of inflow occlusion, which allows parenchymal transection to proceed with less blood loss. A meta-analysis of 11 trials including 669 patients showed no difference in clinical outcomes, including mortality and morbidity. In subgroup analysis, four trials showed no significant differences in ALT levels and five studies showed no differences in AST between IPC and control groups. In their trial of 84 patients undergoing liver resection, Arkadopoulos *et al* report reduced IL-6, IL-8, AST and malondialdehyde levels in the IPC group, with fewer numbers of cells showing signs of apoptosis by terminal dUTP nick-labelling (TUNEL) assay **(210)**.

2.7 Fatty Liver and Ischaemia-Reperfusion Injury

With the increasing prevalence of obesity, fatty liver disease may be present in around a third of the population, and in 20-25% of patients undergoing major liver surgery **(211)**. Steatotic liver grafts have a higher incidence of primary non-function **(212)**. A meta-analysis of 1000 patients undergoing hepatectomy showed an increased risk of postoperative complications (RR 1.53, 95%CI 1.27 to 1.85) and death (RR 2.79, 95%CI 1.19 to 6.51) in patients with at least 30% steatosis compared with no steatosis **(213)**. Given that NAFLD is a marker of the metabolic syndrome, patients with steatosis may have a higher incidence of obesity, cardiopulmonary disease and diabetes **(214)**. These pre-operative factors are all known risk factors of post-operative complications **(215)**. Despite this, Kooby *et al* report in their analysis of 485 patients, steatosis is an independent predictor of complications, and remains a significant independent factor even in a multivariate prediction model that includes co-morbidities **(216)**.

2.8 Bariatric Surgery and NAFLD/NASH

Following bariatric surgery, weight loss leads to reduction in volume of fat depots. The reduction in visceral fat and high circulating lipid levels leads to decreased free fatty acid flux in the liver **(217)**. Thus, intracellular lipid in the hepatocytes can be metabolised or released, leading to reduction or resolution of steatosis. Decrease in insulin resistance correlates with both weight loss and reduction in steatosis **(218)**. At the same time, reduction in fat depots are also associated with reduced production of pro-inflammatory cytokines **(217)**. In theory, this should lead to a reversal of the process of progression from simple steatosis to NASH. Fibrosis however is a non-reversible process, as it involves deposition of collagenous scar tissue within the liver parenchyma. In the longer term, there are case reports of acute liver failure occurring following bariatric surgery, in patients with NASH **(219)** and cirrhosis **(220)**.

In a meta-analysis of 15 studies including 766 paired liver biopsies, 91.6% (95% confidence interval 82.4–97.6%) of patients showed reduction in steatosis after surgery **(221)**. Similarly, NASH was present in 53% of biopsies, and improvement in histological evidence of NASH occurred in 81.3% (95% CI, 61.9%–94.9%). Although 65% of biopsies had evidence of fibrosis, only 121 paired biopsies were systematically scored for fibrosis by a histopathologist. Even so, 65% (95%CI 38.2-88.1%) showed improvement.

In a large prospective study of 381 patients undergoing various forms of weight loss surgery, Mathurin *et al* were able to compare 267 paired liver biopsies at 1 year and 211 at 5 years following surgery. Of 190 patients with a NAS fibrosis score of F0 at baseline, 39 had a score of F1 at 1 year and 41/166 at 5 years. Although NAS, % steatosis and ballooning all reduced at 1 and 5 years, inflammation scores and extent of fibrosis increased. In 99 patients with NASH, mean NAS score reduced from 3.71 ± SEM 0.86, to 2.13 ± 1.48 at 1 year and 1.92 ± 1.56 at 5 years, with significant reductions in steatosis and ballooning, but no significant fall in scores of inflammation or fibrosis **(222)**.

In terms of non-invasive markers, there are no studies where markers are cross-validated with follow up liver biopsy. Diab *et al* studied cytokeratin-18 levels and

found in 34 patients, 3 (8.8%) had an increase in CK-18 levels and 31 (91.2%) had a decrease over 6 months. Median decrease in CK-18 was 44% (range 13 to 88%) **(223)**. These decreases correlated with the fall in BMI and ALT. Kahraman *et al* evaluated a range of biomarkers in 108 patients after bariatric surgery **(224)**. They found significant decreases in CK-18 M30 and M65 concentrations in both NASH and simple steatosis groups, although the relative decreases were much larger in the NASH group.

The overwhelming consensus from available data is that bariatric surgery improves NAFLD/NASH in the medium to long term. However there is a lack of large scale, well designed studies to fully evaluate the proportion of patients whose fibrosis progresses and what the identifiable pre-operative risk factors for this progression **(225)**.

CHAPTER 3

LIVER INJURY AND PNEUMOPERITONEUM

3.1 Introduction

In Chapters 1 and 2, the role of bariatric surgery has been discussed, including its beneficial effect on reducing inflammation and oxidative stress in the morbidly obese. Steatotic livers are particularly vulnerable to ischaemia, infection and other toxic insults. The majority of patients undergoing bariatric surgery have fatty liver disease. There is little work on post-operative inflammatory response and liver injury in this group of patients. This chapter aims to review the existing literature, which focuses on the inflammatory response following laparoscopic surgery. This review will give further context to the original work in this thesis presented in subsequent chapters.

3.2 Liver injury during bariatric surgery

During laparoscopic bariatric surgery, the left lobe of the liver is retracted to expose the stomach. Obese patients have large livers secondary to fatty infiltration. Liver retraction is often performed with a fixed metal bar, called a Nathanson retractor. The shear forces may tear the liver or cause bleeding and haematoma **(226)**. The compression may even cause liver infarction, characterised by pain, elevation of liver enzymes and can even lead to multi-organ failure and death **(227)**. After gastric bypass, Yu *et al* identified three patients out of 72 with asymptomatic left lobe infarction on computed tomography (CT) **(228)**. More commonly, liver retraction merely squeezes the blood supply, which causes the liver to be partially starved of oxygen (ischaemia). Figure 3.1 shows a typical intra-operative finding of an ischaemic-looking purplish left lateral liver segment. When the retractor is released at the end of surgery, the restoration of a normal oxygenated blood supply can also cause tissue damage (ischaemia-reperfusion injury). The combination of mechanical trauma and reperfusion injury is sufficient to cause derangement of post-operative liver transaminases. In gastric bypass, this may be up to a six-fold rise in aspartate transaminase and alanine transaminase

from baseline levels **(229)**. Serum liver enzyme levels return to baseline within 72 hours.

Morris-Stiff *et al* demonstrated that laparoscopic antireflux surgery was associated with a much greater rise in liver enzymes than laparoscopic cholecystectomy (LC), because of the additional effect of a Nathanson retractor, which is not required in LC **(230)**. The phenomenon is not unique to laparoscopic surgery and deranged liver function is seen after open upper abdominal surgery as well **(229, 231)**. In operations where liver retraction is not required, the pneumoperitoneum still has a significant effect on the intraabdominal organs. Since the inception of laparoscopic surgery, there has been much interest in the possible deleterious effects of the pneumoperitoneum on liver function, oxidative stress and immune function. The literature pertaining to this is reviewed herein.

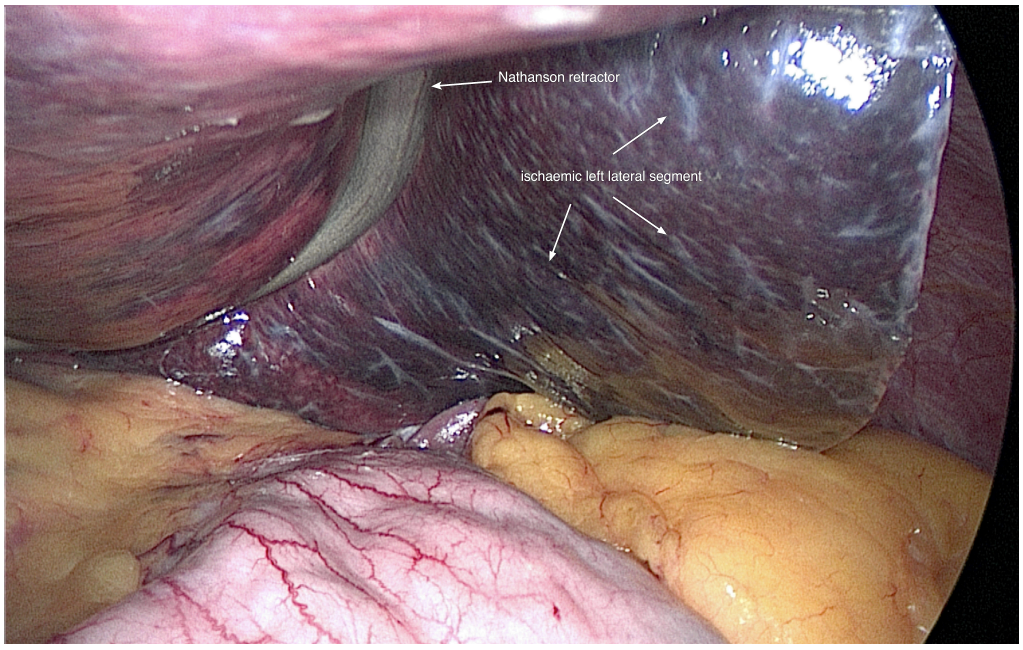


Figure 3.1 Purplish ischaemic appearance of left lateral segment of liver following placement of Nathanson liver retractor during laparoscopic surgery (intraoperative image kindly provided by Mr Ameet Patel)

3.3 Pneumoperitoneum causes liver injury

Studies of the direct effects on liver tissue in humans are more difficult to perform, given the practical and ethical difficulties of obtaining tissue in patients where liver biopsy is not clinically indicated. Three pig models however do shed some light on the matter. Alexakis *et al* performed sequential liver biopsies over the course of a 2 hour pneumoperitoneum in 14 pigs **(232)**. They quantified 11

histological hallmarks of liver injury as a score of 0 to 3. They found that amongst others, scores for intralobular and portal inflammation, oedema, hyperemia, focal necrosis all increased significantly over time, mostly as changes between pre-operative values and biopsies taken at 2 hours or after desufflation. The major drawback of the study is the lack of validation of the scores either internally or externally, with only one pathologist used. All of the mean score values were <1, suggesting that most of the 11 measured parameters were scored either 0 or 1. Yoshida *et al* saw an (unquantified) increase in hepatocyte ballooning and lytic change around the central vein, with sinusoidal dilatation and congestion. Evidence of apoptosis, though TUNEL-staining of hepatocytes, was seen in the pneumoperitoneum group **(233)**. Hoekstra *et al* in a smaller study of 8 pigs found similar histological findings at post-mortem liver biopsy, but no pre-operative comparisons were taken **(234)**. Given the conflicting evidence, the effects of pneumoperitoneum on the splanchnic circulation and liver blood flow may not be the main cause of damage. The changes may be due to direct pressure effects on the liver. Leister *et al* performed intravital video microscopy on the left liver lobe in a rat model of pneumoperitoneum **(235)**. They found that an 8mmHg pneumoperitoneum causes significant decreases in sinusoidal perfusion to 70-80% of baseline, with an 8 fold increase in the numbers of stagnant leucocytes within the sinusoids.

Andrei *et al* reviewed 270 patients undergoing LC and 64 having open cholecystectomy. They found that ALT was deranged in 34% after LC, compared with only 15% after open surgery (p=0.004), concluding that the pneumoperitoneum was the main distinguishing factor between groups **(236)**. Giraudo *et al* studied 63 patients undergoing LC in 3 groups – a gasless group using an abdominal wall lifting device, a 10mmHg pneumoperitoneum group and a 20mmHg pneumoperitoneum group. They found that the rise in ALT after surgery was higher in the pneumoperitoneum groups than the gasless group and was proportional to both the length of surgery and the pressure used **(237)**. Similarly Gupta *et al* showed that a lower pressure pneumoperitoneum decreases the extent of elevation of post-operative liver enzymes **(238)**. At least 16 published studies have established that ALT and AST rises significantly after LC **(239)**. Although liver enzymes may undergo between a two and six-fold rise after laparoscopic

surgery, this is not thought to have clinically significant effects on the majority of healthy non-obese patients undergoing routine surgery **(240)**. Despite attempts to mitigate the extent of liver injury using abdominal lift devices **(241)** rather than insufflation or pneumoperitoneum using other inert (but more expensive) gases **(242)**, only the application of intermittent calf pressure pumps have become standard practice **(243)**. Similarly there is no clear evidence to favour the use of low pressure pneumoperitoneum over standard pressures **(244)**, although most surgeons will keep the pressure as low as technically feasible **(245)**.

3.4 Physiological effects of pneumoperitoneum during surgery

Insufflation of the abdominal cavity with carbon dioxide (CO_2) is required to perform laparoscopic surgery. For patients with good cardiorespiratory function and no other signs of organ dysfunction, pneumoperitoneum has only minor clinically relevant effects. Some CO_2 will dissolve across the peritoneal lining and dissolve in the blood stream, forming bicarbonate and hydrogen ions. These are excreted via lung ventilation. Therefore anaesthetists monitor end tidal CO_2 concentration and arterial blood pH and can increase minute ventilation volumes if CO_2 levels are too high **(246)**. Increased intraabdominal pressure causes splinting of the diaphragm, reducing ventilation of the lung bases and adversely affecting the mechanics of breathing, requiring higher ventilation pressures. These effects are exacerbated in obese patients given the additional weight of their abdominal walls with attached fat aprons **(247)**.

In bariatric surgery, patients are operated on in a steep head-up position. This has an additive effect with the increased intraabdominal pressure on venous return to the heart, causing a decrease in cardiac output. Lower CO_2 pressures are better tolerated **(248)**. Renal blood flow is also reduced by pneumoperitoneum and may result in a fall in urine output and transient deterioration in renal function. This usually recovers within hours of surgery and the effects can be reduced by adequate hydration **(249)**.

3.5 Other pathophysiological effects of pneumoperitoneum during surgery

3.5.1 Splanchnic Hypoperfusion

Both animal and human studies have shown that pneumoperitoneum can cause reduced splanchnic perfusion **(250)**. The main importance of splanchnic hypoperfusion is the potential for a reperfusion phenomenon to occur on release of peritoneum **(251)**.

A combination of decreased cardiac output, increased vascular resistance and direct compression of intra-abdominal vessels is thought to result in reduced blood flow **(252)**. Ishizaki *et al* studied blood flow in the superior mesenteric artery in dogs and demonstrated a reduction in perfusion with a pneumoperitoneum of 16mmHg **(253)**. In a pig model, Agusti *et al* demonstrated a 20-30% reduction in splanchnic blood flow measured by ultrasonic flow probe after induction of a 15mmHg pneumoperitoneum **(254)**. They also showed reduction in intestinal intramucosal perfusion, measured by laser Doppler probe. This was improved by administration of dobutamine to increase cardiac output, although blood flow was unaltered. Schilling *et al* demonstrated dramatic reductions of intraabdominal blood flow after elevation of pneumoperitoneum pressure from 10 to 15mmHg: stomach >40%, jejunum 32%, liver 39% **(255)**.

The liver has a dual blood supply from the hepatic artery and portal vein. Portal vein blood pressure is 5-10mmHg **(256)** and supplies the majority of blood to the liver. Therefore routinely used pneumoperitoneum pressures of 12-15mmHg have a significant impact on hepatic perfusion **(257)**. Clearance of a luminescent dye indocyanine green (ICG) from the plasma by the liver can be used as a non-invasive measure of hepatic blood flow. Eryilmaz *et al* measured ICG clearance in 42 patients before and after LC in two groups with 10mmHg and 14mmHg pneumoperitoneum. Whilst a 10mmHg pneumoperitoneum had minimal impact on ICG clearance before and after surgery, the 14mmHg group demonstrated a significant 25% reduction **(258)**. There was a corresponding significant rise in ALT and AST post-operatively. Eleftheriadis *et al* inserted single fibre laser Doppler probes into the liver parenchyma to measure hepatic perfusion and demonstrated a significant fall during laparoscopy which resolved as soon as the pneumoperitoneum was released **(259)**. Indirect measurement of hepatic

perfusion can be made by measuring hepatic vein blood flow via transoesophageal echocardiography. Meierheinrich *et al* showed that hepatic vein blood flow increased during pneumoperitoneum of 12mmHg, which conflicts with other data **(260)**. This may suggest that this is a poor technique of assessing liver perfusion or may reflect that understanding of the dynamics of liver blood flow is still limited. The results of Goitein *et al* further cloud the issue. In a pig model, which may be more representative of humans than small animal models given the larger size and similar morphology of the abdominal cavity, they measured intestinal flow using coloured microspheres at various time points during pneumoperitoneum. Perfusion was measured as a function of microsphere concentration within the intestinal mucosa. They found that perfusion increased slightly after insufflation and then returned to near baseline levels, suggesting a degree of autoregulation and concluded that 15mmHg pneumoperitoneum has little effect on splanchnic perfusion **(261)**. Helium had no significantly different effects compared with CO_2.

A number of measures have been tried to reduce the effects of the reduced perfusion. Abdominal wall lifting devices are associated with less disturbance to mean arterial pressure, gastric and intestinal mucosal pH, and urine output **(260)**. Schimazutsu *et al* found pneumoperitoneum reduced splanchnic blood flow in a pig model. They determined that this correlated with reduced nitric oxide (NO), a potent vasodilator, and that splanchnic blood flow could be increased back to baseline levels by administration of ethyl nitrite, a NO donor **(262)**. Ali *et al* conducted a similar experiment in pigs using ethyl nitrite, measuring perfusion using laser Doppler. They found that ethyl nitrite corrected hepatic blood to baseline but had minimal impact on renal flow, which was not reduced during pneumoperitoneum **(263)**. Preconditioning (PC) by application of a short period of pneumoperitoneum, followed by release, then reapplication has been studied in a rat model. Yilmaz *et al* showed that markers of liver injury and oxidative stress were reduced by ten minutes of PC **(251)**. Perhaps the most clinically useful and relevant manoeuvre to improve perfusion is application of intermittent calf compression pumps during surgery. Bickel *et al* measured hepatic and renal blood flow using a laparosopic ultrasound Doppler probe during LC. Application of intermittent calf compression reversed the fall in cardiac output induced by

pneumoperitoneum and increased renal and hepatic blood flows significantly compared to controls **(264)**. Urine output also increased in the calf pump group. This manoeuvre has the added benefit of reducing risk of venous thromboembolism and is recommended routinely for patients undergoing laparoscopic surgery **(243)**.

3.5.2 Peritoneal surface changes

The peritoneum comprises a metabolically active layer of secretory cells forming a continuous layer lining the intraabdominal organs **(265)**. The flow of CO_2 during insufflation and maintenance of peritoneum causes dessication and damage to the cells, altering both structure **(266)** and metabolic function. Dissolution of CO_2 to form a weak carbonic acid causes acidosis within the peritoneum. In addition, the increased intraabdominal pressure reduces perfusion and oxygenation of the cells, exacerbating the acidosis **(265)**. Acidosis is likely to provoke an inflammatory response, with increased production of cytokines **(267)** and induction of oxidative stress within the tissues **(255)**. There was much interest in the potential suppression of macrophages and induction of immune dysfunction by CO_2 pneumoperitoneum, although much of the data is conflicting **(265, 267)**. It is clear that the stress response following laparoscopic surgery is smaller than that after open surgery **(268)**, which overall is likely to have metabolic and immunological advantages **(269)**. The suppression of the immune response was postulated to have a potential impact on outcomes after cancer surgery **(267)** but this has not been borne out in longer term clinical studies **(270, 271)**.

3.6 Pneumoperitoneum and oxidative stress

As discussed above, oxidative stress occurs during laparoscopic surgery due to a combination of reduced splanchnic blood flow causing end-organ ischaemia, acidosis within the peritoneum due to dissolution of CO_2 and following evocation of an inflammatory response **(272)**. The difficulties in accurately measuring oxidative stress are discussed in Chapter 4 and it is important to emphasise that the available measures have particular limitations and caveats when interpreting the results.

Animal studies provide greater opportunity for tissue-specific measures of oxidative stress, which may be more reflective of end-organ ischaemia than circulating markers **(268)**. Given the increasing use of laparoscopic living donor nephrectomy as a source of kidney for transplantation **(273)**, animal models of laparoscopic nephrectomy have been developed to examine potential deleterious effects. In a rat model, pneumoperitoneum is associated with increased levels of renal tissue malonaldehyde (MDA) and protein carbonyls (PC) and decreased superoxide dismutase (SOD) **(274)**. Xu *et al* demonstrated a similar increase in MDA and decrease in SOD after 1 hour of pneumoperitoneum in their rat model. They noted differences in histology with evidence of fatty degeneration in the 1 hour group with increased expression of hypoxia-induced factor 1 mRNA in the liver **(275)**. Hypoxia-induced factors are stimulated by anaemia and ischaemia and may act through the NF-κβ pathway, promoting inflammatory cytokines including TNFα **(276)**. Although animal studies clearly demonstrate a rise in oxidative stress after pneumoperitoneum, proportional to the length and/or pressure used, with increased stress compared with open surgery, it may not be possible to extrapolate the effects to humans given the differences in size and anatomy. In contrast to human studies, most animal studies merely examine the effect of pneumoperitoneum and do not include any laparoscopic surgery given the restriction in size **(277)**.

Yiannakapoulou *et al* systematically reviewed the literature for human studies and describe 9 trials demonstrating that laparoscopy induces oxidative stress **(278)**. The studies employ a variety of markers, discussed above, including markers of oxidative stress and antioxidant defense. They demonstrated that lipid peroxidation and protein oxidation occur within hours of surgery. Much of the data is conflicting regarding the duration of the increase in oxidative stress. Sammour *et al* also performed a similar systematic review and described significant heterogeneity in the results. They showed that laparoscopically-induced oxidative stress is less than that seen in open surgery and that the duration of the response lasted for 24 hours in the majority of studies **(257)**.

CHAPTER 4:

THE EFFECT OF INTRAOPERATIVE N-ACETYLCYSTEINE ON HEPATOCELLULAR INJURY DURING LAPAROSCOPIC BARIATRIC SURGERY. A RANDOMISED CONTROLLED TRIAL.

4.1 Introduction

This chapter describes the study protocol for a randomised clinical trial of an investigational medicinal product (CTIMP). The protocol was formally approved by the Medicines and Healthcare products Regulatory Agency (MHRA) of the United Kingdom (UK). The procotol is not reproduced here verbatim. The general format proscribed by the MHRA in reporting CTIMPs has been followed. Additional sections of the formal report of the trial to the MHRA can be found in Appendix 1.

The detailed introduction in Chapter 1 and 2 sets out the relevant background about bariatric surgery, fatty liver disease and ischaemia-reperfusion injury. In Chapter 3, there is a review of the literature on the effect of pneumoperitoneum on the inflammatory response and oxidative stress, including its relevance to the liver. The aim of this chapter was to study the effect of laparoscopic surgery in morbidly obese patients, with NAFLD/NASH, with regards to markers of liver injury and inflammatory response, and on the clinical outcome following surgery. This is within the context of a CTIMP using N-acetylcysteine, an antioxidant used to try to reduce the extent of liver injury. An overview of the clinical and experimental data available on N-acetylcysteine (NAC) in related contexts is given. Much of this introduction also pertains to Chapter 4, where the results of biomarkers of oxidative stress, inflammation and liver injury deriving from this trial are presented.

4.2 Background

4.2.1 Bariatric Surgery, fatty liver disease and post-operative liver dysfunction

Obesity is associated with many complications, including diabetes mellitus, hypertension, dyslipidemia, cardiovascular disease, gallstones and cholecystitis, sleep apnea and other respiratory dysfunction, and the increased incidence of certain cancers **(279, 280)**. Morbidly obese patients have a higher rate and severity of complications after all types of surgery **(281)**. Over 80% of patients undergoing bariatric surgery have fatty liver disease **(282)**. Fatty liver is more vulnerable to damage by toxins, ischaemia and infections, triggering further liver injury or precipitating liver failure. Obesity and fatty liver are independent risk factors for the development of complications after liver resection **(283)**. Fatty liver disease is the fastest growing indication for specialist hepatology referral in the UK **(284)**.

Bariatric surgery is the most effective treatment for morbid obesity **(35)**. However, the liver is subjected to further stress during surgery that may complicate the post-operative recovery. Retraction of the liver during surgery and subsequent release causes ischaemia and then ischaemia-reperfusion injury (IRI), indicated by derangement of post-operative liver function tests, with up to a six-fold rise from baseline levels lasting for up to 72 hours **(229)**. In addition, the pneumoperitoneum created to facilitate laparoscopy also causes a degree of injury to the intra-abdominal viscera. This injury contributes to the systemic inflammatory response and may jeopardise the recovery of the patient. Clinical outcomes may include post-operative fever, renal failure, respiratory distress, hypotension and liver failure **(285)**. Up to 10% of bariatric surgery patients may have clinically significant post-operative complications, increasing hospital stay **(35)**. Upto 18% of patients with pre-existing liver disease have progressive hepatic dysfunction following bariatric surgery, with severity ranging from transient elevation of liver transaminases **(229)** to organ dysfunction **(286)** and rarely liver failure causing death. **(219)** In our own unit, 120 patients underwent weight loss operations in 2006-7. Their average hospital stay was 7 days, with complications affecting 8% of patients. Over 80% had deranged liver function

tests for at least 72 hours post-operatively and 7% suffered infectious complications (unpublished data).

4.2.2 N-acetylcysteine

N-acetylcysteine (NAC) is a pharmacological agent, approved for use in the prevention of radiological contrast induced nephropathy and the management of paracetamol overdose. It is a derivative of the amino acid cysteine and acts as a precursor to glutathione, an important antioxidant **(287)**. Glutathione (GSH) is the most abundant intracellular buffer and mitochondrial antioxidant and made of the amino acids glycine, glutamic acid and cysteine. Upon contact with ROS, oxidised glutathione (GSSH) is produced. This can then be regenerated in a reducing reaction which consumes NADPH by glutathione reductase (shown in Figure 4.1) **(288)**. Hypoxia can induce GSH synthesis, although the rate limiting step is the availability of cysteine and synthesis is an energy-dependent process **(289)**. NAC is a highly soluble cysteine precursor which can easily diffuse into cells, boosting GSH levels rapidly **(289)**.

Figure 4.1 The Glutathione Antioxidant System (after Sastre *et al*, 1996, adapted from Rahman *et al*, 1999) (290)

NAC also acts directly as a free radical scavenger, reacting with hydrogen peroxide **(291)**. It is used widely as a treatment for paracetamol poisoning, where intracellular glutathione is depleted, leading to hepatocellular necrosis and liver failure. Its effects as an anti-oxidant have been established *in vitro* and in animal studies **(291)**

There have been a number of studies looking at the effect of N-acetylcysteine in critical care settings, including randomised controlled trials in severe sepsis **(292)**, coronary artery bypass grafting **(293)** and acute pancreatitis **(294, 295)**. These studies have not shown a significant beneficial clinical effect, although one study demonstrated an increase in hepato-splanchnic blood flow **(296)**. Koca *et al* compared the effect of NAC and ischaemic preconditioning on markers of oxidative stress in patients undergoing arthroscopic knee surgery under tourniquet control **(297)**. They found that both treatments were effective in significantly reducing oxidative stress, with lower MDA levels, increased superoxide dismutase and glutathione peroxidase levels and an increase in total antioxidant capacity compared with control group.

4.2.3 NAC in Liver Surgery

A number of animal models of ischaemia-reperfusion injury (IRI) have investigated the efficacy of NAC in reducing hepatocellular injury. These studies have variously shown a number of beneficial effects including increased bile flow, improved sinusoidal blood flow, decreased post-operative liver transaminases and reduced evidence of injury on liver histopathology **(298)**. Studies in pig models have shown a reduction in post-IRI inflammatory markers and free radical production, whilst isolated porcine liver perfusion studies have shown better Kupffer cell function following NAC administration **(299)**. In nine clinical trials in human orthotopic liver transplantation, NAC was administered either to donors before organ retrieval or to recipients before graft revascularisation. Three trials showed a statistically significant reduction in post-operative liver enzymes, although the clinical significance in terms of complications and graft survival could not be evaluated in these small studies **(299)**. Two studies of NAC in liver resection have been performed, including a total of 119 patients **(299, 300)**. Robinson *et al* found no differences in clinical outcomes between groups, though day 3 ALT levels were lower in the treatment group (Controls 308 (range 91–1599 versus NAC 223 (74–764); $p < 0.01$) **(300)**. Worryingly the NAC group had a prolonged prothrombin time on post-operative day three, although this was of doubtful clinical significance.

All of these reported studies have reported on small numbers of patients and were underpowered to detect effects on clinical outcomes. However, these small trials have not been performed specifically in an obese population. No sub analyses looking specifically at obese patients have been possible. Nevertheless, a number of liver surgery centres continue to champion the use of NAC based on the experimental evidence.

4.2.4 NAC in fatty liver disease

Progression of fatty liver disease to steatohepatitis and fibrosis occurs due to oxidative stress and chronic inflammation **(125)**. Steatotic hepatocytes are more vulnerable to toxic insults and hypoxia, being less able to tolerate the accompanying mitochondrial dysfunction and buffer the increased oxidative stress **(181)**. Thus, potential antioxidant and anti-inflammatory effects of NAC are an attractive treatment for patients with NAFLD **(301)**.

In a rat model of NASH, an oral formulation of NAC given in feeds was associated with a 34% reduction in the area of collagen deposition seen on liver histopathology compared with the control group **(302)**. This reduction was accompanied by a corresponding changes in expression of genes involved in fibrogenesis, including matrix metalloproteinases, and downregulation of heat-shock proteins. NAC fed to rats decreased body fat percentage and lowered serum triglycerides and leptin, compared to controls and lowered hepatic stearoyl-coenzyme desaturase-1 (SCD1), a key enzyme of fatty acid and energy metabolism **(303)**. In rats fed with a hepatotoxic compound known to cause oxidative stress and hepatic steatosis, NAC supplementation decreased hepatocyte lipid accumulation, as assessed on microscopy **(304)**. In a rat model where overfeeding polyunsaturated fats leads to NASH, NAC supplementation was associated with lower liver pathology scores, reduced markers of oxidative stress within the liver and decreased hepatic TNFα mRNA expression **(305)**. In an obese ob/ob mouse model, administration of NAC for 60 days decreased the degree of steatosis and lipid peroxidation in the group fed a methionine-choline deficient diet, which induces NASH, and a high-fat diet, which induces steatosis **(306)**. Similar results were seen in another high-fat diet rat model of NASH, where NAC

supplementation reduced steatosis and necroinflammatory scores, along with increased tissue GSH levels **(307)**.

In a partial hepatectomy model in rats with NAFLD, pre-operative administration of NAC was associated with lower levels of malondialdehyde, a marker of lipid peroxidation and higher mitotic indices and proliferating cell antigen levels in the remnant liver after 48 hours, suggesting NAC may improve liver regeneration **(308)**. In an ischaemia-reperfusion rabbit model with steatosis, administration of NAC before reperfusion was associated with decreased ALT levels, used as a marker of liver injury, and improved hepatic microcirculation, as evaluated by laser Doppler flow **(309)**. In a rat model of ischaemia-reperfusion, the effect of NAC was compared in steatotic and normal livers. Administration of NAC in the normal liver rats had no effect on markers of oxidative stress and histopathological evidence of liver injury. However, in the steatotic rats, there was a significant reduction in hepatic MDA, lung myeloperoxidase and liver injury scores **(310)**. NAC was given in combination with other drugs in a rat model of IRI, which had a significant effect on reducing post-IRI transaminases, MDA and inflammatory response **(311)**.

Human studies of NAC in the context of NAFLD/NASH are less encouraging. Pamuk *et al* gave 18 patients with NASH daily oral NAC supplements for 4 weeks and noted a reduction in ALT and AST, although their control group also had a similar reduction in transaminases over the same time period **(312)**. Khoshbaten *et al* randomised 30 patients with NAFLD to receive NAC or Vitamin C for 3 months **(313)**. Serum ALT fell significantly in the NAC group from 77 to 51.7 ($p=0.014$). However, ALT in the Vitamin C group was significantly lower at baseline. The study also showed a statistically significant 5mm reduction in the size of the spleen in the NAC group, which the authors interpret as a possible sign of reduction in steatosis.

In our unit, NAC has been shown to improve the metabolic function of hepatocytes when given as an infusion in fatty livers rejected for orthotopic transplantation **(314)**. Comparing the effect of NAC added to a perfusion buffer used to isolate hepatocytes from steatotic livers to the standard buffer (n= 5 and

5), cells from the NAC group had a significantly higher mean viability (81.1 ± 1.7% vs. 66.0 ± 4.7%; p = 0.003). The quality of isolated hepatocytes was assessed using a cell attachment assay, a protein synthesis assay and measurement of mitochondrial dehydrogenase activity, which were all significantly higher in the NAC group. Although the mechanism by which NAC had a beneficial effect was not elucidated, other cell models have shown that NAC reduces inducible nitric oxide synthase (iNOS), perhaps by inhibiting cytokine-stimulated iNOS expression **(315, 316)**.

4.2.5 Why perform this randomised trial?

The consensus is that bariatric surgery can improve fatty liver disease **(282)**. However, it is possible that liver damage may worsen transiently when the liver suffers ischaemia intra-operatively. This injury can trigger a systemic inflammatory response and jeopardise the recovery of the patient. The directly attributable clinical effect of intraoperative hepatocellular damage on post-bariatric surgery outcomes has not been quantified. Although usually self-limiting, a larger post-operative inflammatory response may predispose the patient to a worse response to further insults, such as anastomotic leakage or post-operative infection – a so-called "second hit" **(317)**. Clinical outcomes may include post-operative pyrexia, and rarely to more serious problems, such as renal failure, acute respiratory distress syndrome and hypotension. Up to 10% of bariatric surgery patients may have clinically significant post-operative complications **(35)**, leading to additional allocation of health resources and an increase in hospital stay. Obesity and steatosis are independent risk factors for the development of complications after liver resection **(283)** and transplantation **(318)**. In the longer term, there are case reports of acute liver failure occurring following bariatric surgery, in patients with NASH **(219)** and cirrhosis **(220)**. Follow-up studies with post-operative liver biopsies have confirmed resolution of steatosis, but with varying changes in the degree of inflammation and fibrosis **(282)**.

No study has specifically looked at the role of perioperative N-acetylcysteine in a morbidly obese population. These patients have a propensity to develop an exaggerated inflammatory response to surgical trauma **(285)** and are more

vulnerable to oxidative stress **(129)**. With an increasing proportion of the population being obese, the prevalence of obesity-related post-operative complications will escalate. N-acetylcysteine's role as an anti-oxidant and free radical scavenger may have a protective effect within this population, ameliorating the intra-operative insult to the fatty liver. *In vitro* data demonstrate a significant benefit from N-acetylcysteine for hepatocytes derived from fatty livers **(314)**. *In vivo*, the effect of NAC may reduce immediate post-operative complications and also prevent further deterioration long term in the subset of patients with NASH. The use of NAC may be extended to other obese patients undergoing liver surgery, reducing the burden of post-operative complications on the health service.

4.3 Aims and Outcome Measures

This study aims to answer the following question:

- Does administration of NAC reduce the surgical insult, especially signs of hepatocellular injury?

The following outcome measures are used to quantify the decrease in extent of hepatocellular damage and its clinical effect of NAC on the treatment group as measured by:

- Difference in liver function tests before and after surgery, specifically AST and ALT as markers of hepatocellular damage (ALT is more specific to liver)
- Differences in post-operative morbidity and length of hospital stay
- Extent of hepatocellular damage, as assessed by histopathological criteria

4.4 Methodology

4.4.1 Trial Design and Patients Recruitment

This was a prospective randomised controlled trial investigating the effects of N-acetylcysteine, performed between July 2009 and August 2012. Full review by the Local Reseach Ethics Committee at King's College Hospital and approval by the MHRA (UK) was sought and obtained before commencement of recruitment (see Appendix 2, Section **B1.1** for Sponsor details).

There are two study groups:

1. Treatment Arm – NAC group
2. Control Arm

Patients were seen in the Multidisciplinary Morbid Obesity Clinic or Pre-operative Assessment Clinic at King's College Hospital, who were referred for morbid obesity surgery.

Fully informed, written consent obtained prior to enrolment into this study.

Inclusion criteria were:-

Male or Female

Age range 18 to 75 years inclusive

Patients must meet the criteria set out by NICE for morbid obesity surgery, that is they must have BMI >40kg/m2 or >35kg/m2 with obesity-related complications, and are undergoing either Laparoscopic Adjustable Gastric Banding or Roux-en-Y Gastric Bypass or Laparoscopic Sleeve Gastrectomy

Exclusion criteria were:-

Patients undergoing Duodenal Switch

Patients undergoing OPEN bariatric surgery (a small minority of total patients)

Pregnancy

History of chronic liver disease, including viral hepatitis, haemochromatosis, alcoholic liver disease or known alcohol intake 28 units per week (and a set of normal tests of liver disease screening including negative hepatitis serology and normal autoantibody screen)

Previous liver surgery, eg resection, orthotopic transplantation

History of active psychiatric illness, including severe depression, bipolar disorder, schizophrenia and eating disorders
Bleeding tendency or anticoagulant medications
Known allergies to N-acetylcysteine or related compounds

4.4.2 Randomisation and Blinding

Participants were allocated to NAC or Control groups using a sequential sealed envelope method. The research team opened the allocation envelope a minimum of 2 hours before surgery to allow time for Pharmacy to dispense the NAC infusion.

The operating surgeon and anaesthetist were aware if the patient receives NAC infusion as it is easily distinguished due to its bright colour and pungent odour.
The patients were blinded to the intervention.

4.4.3 Trial interventions:

4.4.3.1 Intervention Group - NAC

Subjects randomised to the intervention received N-acetyl cysteine infusion, at a standard 150mg/kg in 200ml 5% dextrose over 15mins at induction of anaesthesia followed by an infusion of 50mg/kg in 500mls of 5% dextrose during surgical retraction of liver for a maximum of 4 hours, together with standard anaesthetic medications.
The maximum dose was limited to 16.5g for loading dose and 5.5g for infusion, making a maximum total dose of 22g per study participant.

The Investigational Medicinal Product (IMP), N-acetylcysteine was sourced as Acetylcysteine 200mg/ml and supplied from Aurum Pharmaceuticals Ltd, Bampton Road, Harold Hill, Romford, Essex RM3 8UG Product License/ UK Marketing Authorisation: 12064/0026.
IMP was handled according to the Medicines for Human Use (Clinical Trial) Regulations 2004 and amended in 2006.

The control subjects received standard anaesthetic medications alone. Anaesthesia was provided by two bariatric anaesthetists, using a combination of propofol and sevofluorane for anaesthesia, with remifentanil and fentanyl for analgesia.

4.4.3.2 Standard Treatment of patients

Patients were treated as per routine practice. Although the inclusion criteria included adjustable gastric banding and Roux-en-Y gastric bypass, only patients undergoing Laparoscopic Sleeve Gastrectomy were included in this study. This was because a concurrent study in the unit was recruiting patients undergoing gastric bypass, so none could be recruited before the closure of the study. The LSG was performed by a single experienced surgeon (Ameet Patel, CI) for all patients. Six trocars were utilised and LSG was performed with standard techniques using a 38-Fr bougie. A 12mmHg carbon dioxide pneumoperitoneum was created. A liver biopsy from the left lateral section of the liver was taken using a Tru-Cut cannula, taking 2 or 3 passes in order to obtain cores of liver tissue at a minimum of 1cm in length. A fixed Nathanson liver retractor was placed under the left lateral section of the liver to facilitate clear visualisation of the hiatus. After devascularisation of the greater curvature using an ultrasonic coagulating dissector, the LSG commenced 4–6 cm proximal to the pylorus on the greater curvature and continued towards the angle of His. The completed staple line was reinforced with absorbable sutures. A further liver biopsy from the left lateral section was taken before release of pneumoperitoneum. Haemostasis was ensured before the operation was completed. Blood loss was recorded by measuring volume of effluent in the suction lavage apparatus. Post operatively, all patients received anticoagulation therapy with low molecular weight heparin and wore compression stockings for embolism prophylaxis.

Gastrograffin meal contrast radiography was performed for all patients and methylene blue dye tests were completed on patients with post-operative drains in situ to detect gastric leak. Qualified dieticians provided post-operative dietary education. Post-operative diet consisted of fluids only for 4 weeks, puréed consistency foods for a further 2–4 weeks, followed by soft foods for an additional 4 weeks, then the gradual return to normal foods in reduced portions.

4.4.4 Sample Collection

All of the study samples were stored in a secure freezer within the Institute of Liver Studies, according to the guidelines set out in the Human Tissue Act (2004).

4.4.4.1 Serum and Plasma

Serial fasting serum and plasma samples were taken:-

(i) before surgery
(ii) at end of surgery
(iii) on post-operative days 1, 2, 3 and 4
(iv) at outpatient follow up 6 months after surgery (see Chapter 4).

Blood samples were taken routinely at these time points for standard biochemical and haematological testing. The following equipment is needed to take the samples: non-sterile gloves, 2 purple top EDTA containing bottles, 1 gold top bottle containing separator gel, 1 plain brown top bottle, standard venesection equipment, including tourniquet, alcohol sterile wipes, green needles and 20mls syringe. Blood samples were sent as per standard clinical practice to the King's College Hospital Haematology and Biochemistry Laboratories for evaluation of routine blood counts and biochemistry analysis.

A further 10 mls of blood was taken as part of the study protocol. The blood was divided equally, with 5mls put into an ice-chilled purple-top bottle, containing EDTA, and 5mls in a yellow-top bottle containing gel separator. The bottles were gently shaken for 10 seconds. The yellow-top bottle was left on ice to allow the blood to clot. In the meantime, the purple-top bottle was centrifuged at 2000g for 15mins and the supernatant was transferred to labelled cryotubes in 0.5ml aliquots and placed immediately into the freezer at -80°C. As soon as the blood had clotted, the yellow-top bottles were centrifuged at 1500g for 15min. Again, the serum supernatant was transferred to cryotubes in 0.5ml aliquots and placed in the -80°C freezer.

All samples were clearly marked with identifiers and stored in the -80°C freezer, which was securely locked and was situated within the laboratories of the Institute of Liver Studies for future analysis.

4.4.4.2 Liver Tissue

Liver biopsies were taken from the left lobe of liver

(i) intraoperatively before onset of liver retraction

(ii) approximately 10 minutes after the release of liver retraction.

Samples were taken by the operating surgeon using a 16Guage Tru-Cut® spring-loaded biopsy needle (UK Medical Limited, Sheffield, UK). Either 2 or 3 passes of the needle through the epigastric port site were required to obtain sufficient tissue.

Needle biopsy of the liver is performed routinely as part of our standard practice to ascertain the extent of fatty liver disease and to diagnose steatohepatitis (NASH).

The following equipment was required for processing the samples: a sterile scalpel for dividing the samples, sterile forceps for handling the samples, a flask with liquid nitrogen (obtained from the laboratories in Institute of Liver Studies), 6 cryotubes (1.8mls size), 2 screw-top histopathology containers with formalin, 2 dry small sterile containers and fine marker pen.

The samples were divided into two:-

(1) a core of liver tissue measuring approximately 1cm in length if possible was fixed immediately in formalin for histopathology evaluation.

(2) the remainder of the liver tissue was divided into 0.5cm lengths and each was fragment was placed in a cryotube and snap frozen in liquid nitrogen immediately and stored at –80 °C in the secure freezer within the Institute of Liver Studies.

4.4.5 Efficacy Variables

The following patient specific data were collected as part of the study.

4.4.5.1 Patient Demographics and medical history

Age, gender and ethnicity

A complete medical history was recorded in the patient's medical notes.

4.4.5.2 Clinical data

Pre-operative: weight, height, BMI, Drug History, physical examination and vital signs

Operative: type of operation, time of retraction, operating time, blood loss, anaesthetic and operative complications

Post-operative: Length of stay, complications

Clinical observations, including haemodynamic parameters, urine output

Follow-up: weight, change in medications, complications (see Appendix 1)

changes in physical examination and vital signs

Although all of the above data was collected in the Case Report Forms, at the time of analysis it was felt that the most important outcome variables in analysis of trial efficacy related to change in BMI, operative details, length of stay and post-operative complications. Concomitant medications were recorded carefully in case of any obvious patterns of adverse events or drug interactions. However, none of these were evident so a detailed analysis of concomitant medications is not provided herein.

4.4.5.3 Specimen data

4.4.5.3.1 Serum and Plasma

- Biochemistry AST, ALT, CRP
  Fasting glucose and insulin
- Haematology WCC, PLT

The above were performed by laboratory staff according to standard techniques by the King's College Hospital Biochemistry Department, on an automated multi-analyser, the Advia 2400 (Siemens Healthcare Diagnostics, Camberley, Surrey, UK). The most important markers of hepatocellular damage and inflammatory change included ALT, AST, CRP, White cell count and platelet count. These outcomes are analysed herein. Changes in insulin resistance, measured by HOMA index, are also described in Chapter 4. Other elements of liver function, such as bilirubin, albumin and INR, were not analysed in this study as they were not appropriate outcome measures in this study.

Insulin resistance is calculated using the Homeostasis Model of Assessment (HOMA)-IR index: $\frac{\text{Fasting glucose (mmol/l) x fasting insulin (mU/l)}}{22.5}$ **(319)**.

4.4.5.3.2 Liver Tissue

- Histopathology NAFLD score

Each liver biopsy specimen was individually marked with an anonymised identification code. Sections were paraffin fixed, embedded and stained with haematoxylin and eosin, reticulin, Orcein and Perl's stains by the Histopathology Department at Institute of Liver Studies, King's College Hospital.

Dr Alberto Quaglia, Consultant Liver Histopathology at King's College Hospital, performed the histopathological assessments, blinded to patient identities, clinical details or timing of biopsies.

4.4.5.3.2.1 Histopathology

A diagnosis of NAFLD was made by a hepato-histopathologist on the basis of typical histological findings in the appropriate clinical setting and following exclusion of other liver disease. The pathologist also classified the biopsy as consistent with NASH or as NAFLD without evidence of NASH.

NAFLD Activity Scoring was undertaken, according to the criteria set out by Kleiner and Brunt **(162)**, defined as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2); thus ranging from 0 to 8.

Scores ≥5 are compatible with a diagnosis of steatohepatitis. Fibrosis was scored 0-4 according to the Kleiner-Brunt definitions **(162)**.

4.4.5.3.2.2 Liver Injury Score

In order to quantify the extent of hepatocellular injury, a local scoring system was formulated and used. There are no validated scoring systems to look at acute liver injury in human livers in this context. Published scoring systems used in animal models of ischaemia-reperfusion injury include the Suzuki score, a score used by Alexakis *et al* to grade pneumoperitoneum-induced liver injury in a pig model and a score developed in a multi-centre international study originally to look at chemotherapy-induced liver injury. The Suzuki score is the sum of histological changes that are scored 0-4 on the degree of cytoplasmic vacuolation, sinusoidal congestion and necrosis of parenchymal cells **(320)**. Alexakis *et al* scored the following 11 features 0-3: portal inflammation, intralobular inflammation, eosinophilic infiltration, edema, sinusoidal dilatation, sinusoidal hyperemia, centrilobular dilation, centrilobular hyperemia, pericentrilobular ischemia, focal lytic necrosis, and confluent necrosis **(232)**.

Rubbia-Brandt graded the following features: steatosis, sinusoidal dilation, nodular regeneration, centrilobular or portal vein lesions, centrilobular vein and perisinusoidal fibrosis, peliosis, perisinusoidal haemorrhage and hepatocellular changes **(321)**.

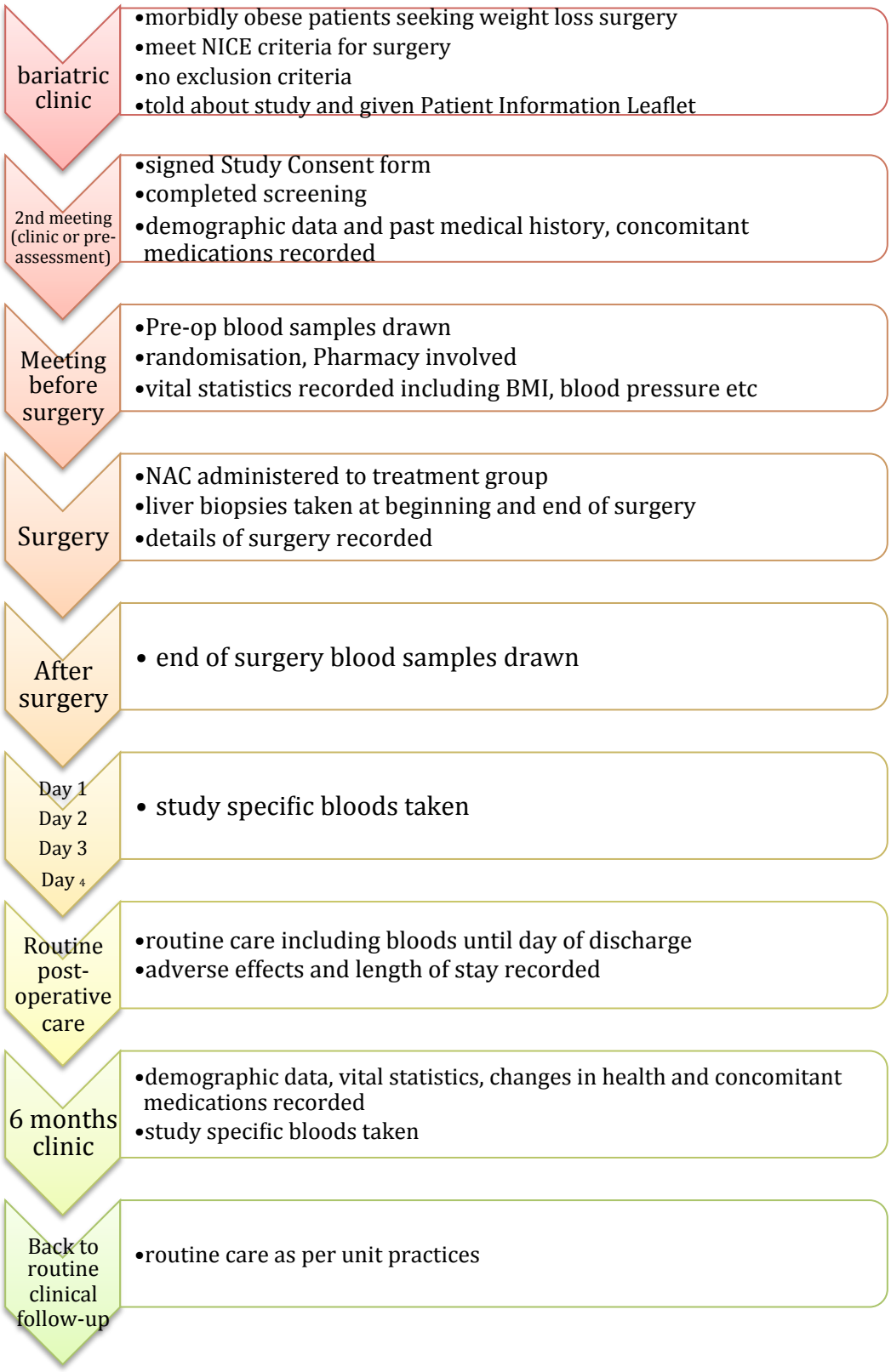
A local Liver Injury Score was developed for this study, based on the NASH Clinical Research Network Scoring System Definitions set out by Kleiner *et al* **(162)**, with some modifications to include other potentially pertinent histological changes (see Table 4.1).

Table 4.1 Parameters assessed in liver biopsy

Item	Description	Score
1. Steatosis (NAS)	<5%	0
	5-33%	1
	>33-66%	2
	>66%	3
2. Ballooning (NAS)	None	0
	Few balloon cells	1
	Many cells/prominent ballooning	2
3. Lobular Inflammation (NAS)	No foci	0
	<2 foci /200X field	1
	2-4 foci /200X field	2
	>4 foci /200X field	3
4. Fibrosis	None	0
	Perisinusoidal or periportal	1
	Perisinusoidal and portal/periportal	2
	Bridging fibrosis	3
	Cirrhosis	4
5. Portal Inflammation	None to minimal	0
	Greater than minimal	1
6. Neutrophil clusters	None	0
	Occasional, after scrupulous search	1
	Scattered, easily seen	2
	Scattered in most lobules	3
	On the verge of confluence, or with confluence elsewhere	4
	Dense clusters	5
7. Eosinophilic infiltrate	None	0
	Occasional, after scrupulous search	1
	Scattered, easily seen	2
	Scattered in most lobules	3
	On the verge of confluence, or with confluence elsewhere	4
	Dense clusters	5
8. Necrosis	Absent	0
	Present	1
9. Mitosis	Absent	0
	Present (look in 10 high power fields)	1
10. Canalicular cholestasis	Absent	0
	Present	1
11. Cholangitis	Absent	0
	Present	1
12. Ductular cholestasis	Absent	0
	Present	1
13. Sinusoidal dilatation	Absent	0
	Present	1
14. Congestion	Absent	0
	Present	1
15. Siderosis	Absent	0
	Present	1
16. Nuclear Vacuolation	Absent	0
	Present	1

NAFLD Activity Score (1) + (2) + (3)
Liver Injury Score (3) + Sum [(5) to (16)]

Figure 4.2 Summary flowchart of study-specific interventions



4.4.6 Safety Variables

One of two experienced Consultant Anaesthetists looked after participants intra-operatively for the duration of their general anaesthetic. They had continuous monitoring of vital signs, including cardiorespiratory parameters, together with the clinical status of the patient. Medications used for general anaesthesia included remifentanil, sevofluorane, fentanyl and morphine, rocuronium, glycopyrrolate and propofol.

Post-operatively, participants were nursed on either a High-dependency Unit or General Surgery Ward by an experienced medical and nursing team, under the supervision of a Consultant General Surgeon in King's College Hospital. There was round-the-clock full access to a range of support services, including radiologists and critical care physicians.

The ward nursing and medical teams continuously supervised patients' recovery. Daily morning ward rounds were conducted, as well as ad-hoc reviews as appropriate. Daily monitoring of renal and liver function and haematological indices was performed as part of standard care.

Where adverse events occurred, the supervising Consultant Surgeon was made aware of any problems and directly involved in the response to these issues, as and when they arose.

Adverse events were defined in advance in the protocol and are listed in Appendix 2.

4.4.7 Adverse events

N-acetylcysteine has a good safety profile. Hypersensitivity reactions, including transient rash and bronchospasm, are recognised. Participants in the treatment will be under general anaesthetic when they receive N-acetylcysteine infusion.

Weight loss surgery carries a risk of complications (approximately 10%). The commonest complications include post-operative chest infection, wound site infection, haemorrhage and anastomotic leak. See Appendix **B1.3** for full list.
These were recorded as Adverse Events. They were not reported as SAEs unless they fulfilled the criteria for SAEs.

4.4.7.1 Treatment Stopping Rules

Individual participants who developed a serious adverse reaction to N-acetylcysteine during the infusion, had the infusion terminated. This occurred in one patient, whose demographic data is included in the final analysis of the trial, but whose other laboratory data is not included as she did not undergo any of the other interventions.

4.4.8 Data Quality Assurance

Outcome measures were recorded where appropriate in the case report forms. Laboratory values of biomarkers and other measures were recorded on Excel Spreadsheets using anonymised patient identification numbers and timepoints. All recordings were made contemporaneously. Cross checking of data points with the original machine outputs in the laboratory was performed in conjunction with laboratory colleagues. No external auditing process or data verification was undertaken as it was felt that the overall volume of data was of a small and manageable scale. In any case, there was no funding source for such third party involvement.

4.4.9 Data Handling and Storage

Data was stored on a Microsoft Excel Spreadsheet.

Personal data was stored securely on password-protected workstations within the office of the Principal Investigator at King's College Hospital and was only accessible to the research team. All data were anonymised before analysis.

Patients' samples were stored in a coded, anonymised form within the Institute of Liver Studies, according to the guidelines set out by the Human Tissue Act (2004).

4.4.10 Sample size calculation

In a previous study, the AST increased significantly from baseline (24±6) and peaked at 24 hours after laparoscopic (152 SD± 102 U/L) and open (231 SD± 518 U/L) RYGB but there was no significant difference in AST levels between study groups[Nguyen *et al*, 2003 **(229)**].

In order to conduct a study with 80% power to detect a 50% reduction in the post-operative rise in AST in relation to placebo in the population of laparoscopy patients, assuming an increase in AST level of 128 U/L and a pooled standard deviation of 100 U/L, the 50% reduction corresponds to an effect of size 0.64 which yields a sample size of 40 patients in each of the two groups, that is 40 patients in control and 40 NAC-infused patients.

4.4.11 Statistical Analysis

Statistical analyses were performed using SPSS 20 (SPSS, USA).
A planned interim analysis was performed after 2 years, at which point it became apparent that various methodological flaws in the design of the trial meant that a meaningful result would be unlikely, even if more patients were enrolled. The reasons for this conclusion are discussed below.

Demographic data and intra-operative variables were compared between groups using independent samples t-test for normally distributed data and Fisher's exact test for dichotomous categorical variables. Complications between groups were compared using Pearson-Chi square test.

The assumption of normality was tested for ALT, AST, WCC, Platelets and CRP at baseline using the Shapiro-Wilk's test ($p>0.05$). These parameters were normally distributed at baseline. Outliers were also found in many of the parameters, as assessed by inspection of a boxplot. These outliers were left in the comparison. Two-way ANOVA using time and treatment group as fixed factors was not possible as the variances were unequal. Thus, independent samples t-tests were used to compare values between groups at the various timepoints, with significance set at $p<0.05$. A secondary analysis of changes in parameters over time within groups was performed using repeated measures ANOVA. Post-hoc comparisons between different timepoints were performed after applying a Bonferroni correction.

Area under curve (AUC) for each parameter was also calculated to give a more general measure of the post-operative change. AUC was estimated using the trapezoid rule, dividing each timepoint into a series of strips, calculating the area of the strips (average height X width, which was set at 1), and calculating the sum of the areas **(322)**. The assumption of normality was satisfied using the Shapiro-Wilk's test and the independent samples t-test was employed to assess differences between groups.

For completeness, a sensitivity analysis was employed by comparing the groups using non-parametric Mann-Whitney-U tests, and independent samples t-test with and without the removal of outliers. The results were similar to the parametric

test outcomes with outliers left. Therefore, results of statistical tests are from parametric tests for ALT, AST, WCC, Platelets and CRP.

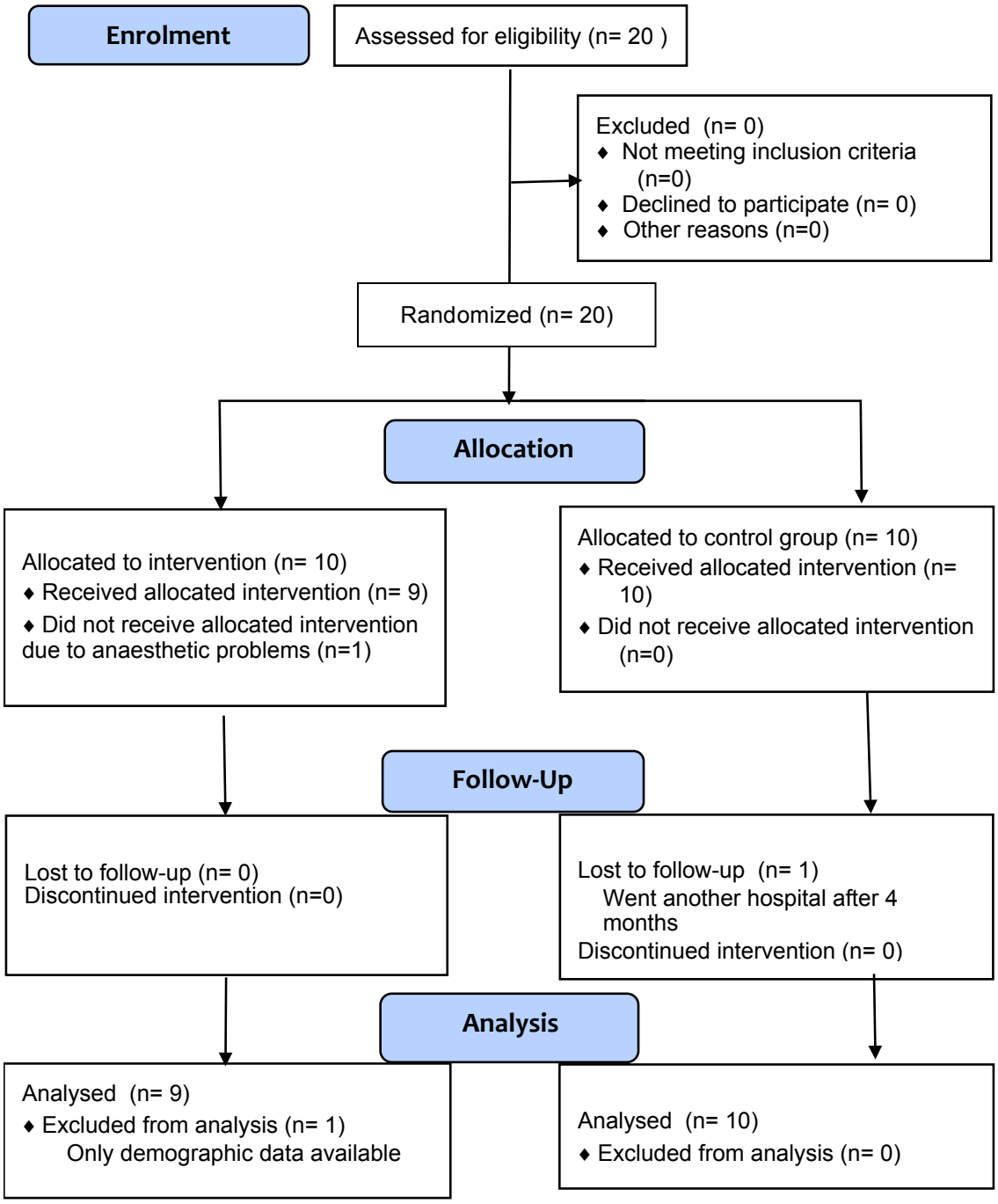
Liver histopathology scores had a normal distribution at baseline (Shapiro-Wilk's test $p<0.05$), and the differences in scores between start and end of surgery were also normally distributed. So independent samples t-test was used to compare the effects of NAC on injury scores. A secondary analysis of change scores within subjects from start to end of surgery was performed using paired samples t-test.

Correlations were performed to assess the interaction between demographic and intraoperative factors and the primary outcome measures. Linearity between co-factors was assessed visually using scatterplots and the relationships were non-linear but monotonic. Therefore Spearman's rank correlation was used. Log10 transformation was used for non-normally distributed variables and Pearson's correlation was performed.

Clinical significance was determined if $p<0.05$.

4.5 Results

Figure 4.3 Flow Diagram of patients through the trial



(according to the Consolidated Standards of Reporting Trials Statement) **(323)**

4.5.1 Demographic Data

20 patients participated in this study. Their baseline characteristics, including age, BMI and co-morbidities were similar. 14 females and 6 males were included, with 2 patients of African-Caribbean ethnic origin in the control group, with the rest of the patients of Caucasian ethnic background. All recruited patients underwent Laparoscopic Sleeve Gastrectomy. By the time recruitment to the study had started, the majority of laparoscopic gastric banding operations were performed as day-case surgery. Any such operations performed as inpatient surgery were for patients with high-risk co-morbidities, not suitable for trial recruitment. Gastric Bypass patients were being recruited to another study in the unit and therefore we were only able to recruit LSG patients. Their BMIs were in the "super" morbid obese range, with an overall mean BMI 60.6 kg/m^2. Ethnic and gender mix were not significantly different. All patients had minimal regular alcohol intake, with 1 patient having given up heavy alcohol intake more than a decade previously. Only 1 patient was a smoker. Tables 4.2 and 4.3 summarise the baseline characteristics of the two groups.

Table 4.2 Baseline Demographic Characteristics of study population

Treatment Group	Control (n=10)	NAC (n=10)
Age (years)	40.70 ±4.3	48.2 ±3.7
Height (cm)	171.9 ±8.4	164.8 ±9.2
Weight (cm)	167.9 ±2.8	166.8 ±4.1
BMI (kg/m2)	61.2 ±3.2	60.4 ±3.9

Mean ± SEM; No significant differences between groups.

Table 4.3 Baseline qualitative characteristics of study population

	Control (n=10)	NAC (n=10)
Female	7	7
Caucasian race	8	10
Diabetes	5	5
OSA	7	8
Depression	4	4
Poor Mobility	2	4
Hypertension	7	7
Asthma	3	5
Cardiac	2	4
High cholesterol	5	6

Qualitative make-up of each group was compared by Chi-square test. No significant differences between groups.

4.5.2 Clinical Outcomes

4.5.2.1 Intraoperative Outcomes

Operating times were similar in both groups (Control mean 134.3 mins SEM ±17.2 vs NAC mean 121. ±8.3, p=0.543). Liver retraction times and recorded estimated blood loss were also similar. A summary is given below in Table 4.4.

Table 4.4 Intraoperative Parameters

	Control (n=10)	Mean (n=9)
Liver Retraction Time (min)	94.6 ±12.6	86.1 ±8.4
Operation Time (min)	134.3 ±17.2	121.9 ±8.8
Blood Loss (ml)	165 ±40.2	216.7 ±35.4
Length of Hospital Stay (days)	7.1 ±0.7	7.0 ±1.0

Mean ± SEM; No significant differences between groups

4.5.2.2 Serious Adverse Events

Complications are classified and tabulated according to the Clavien-Dindo grading in Table 4.5 **(324)**. Of 20 patients, 10 patients had significant complications, meeting criteria for Serious Adverse Events. 1 patient had a post-operative haemorrhage requiring further surgery on the evening of LSG, followed by readmission and further surgery for a staple line failure on post-operative day 13. He was treated in-hospital for a further 8 weeks to allow the leakage and intra-abdominal infection to resolve. 1 patient required a 2-unit blood transfusion post-operatively, after a fall in haemoglobin on the evening after surgery. 1 patient was treated in with non-invasive ventilator support on the high dependency unit for post-operative respiratory difficulties. This was predicted pre-operatively given the patient's history of obesity hypoventilation syndrome and severe sleep apnoea and resolved within 48 hours without significant sequelae. 4 more patients were readmitted within 30 days of discharge. 1 patient became dehydrated following an episode of diarrhoeal infection and was readmitted for rehydration. 2 patients were also readmitted over concerns about poor oral intake and were rehydrated intravenously. 1 patient fainted on post-operative day 22 and was readmitted as a precaution. Pre-operatively, that patient had been taking three antihypertensive medications and these were stopped following readmission.
1 patient was readmitted 3 months following surgery with poor oral intake. Following a period of rehydration, she was discharged but was subsequently admitted two weeks later to a hospital elsewhere with confusion and leg weakness and was diagnosed with Wernicke's encephalopathy, brought on by poor nutritional intake.

2 further patients were reported as Serious Adverse Events, although they did not suffer complications per se. 1 patient was admitted to a different hospital to have an ovarian cystectomy 4 months post LSG. Another patient fell pregnant 5 months post LSG and went on to have an uncomplicated pregnancy and gave birth to a healthy baby.

1 patient in the treatment group developed respiratory difficulties after intubation, with evidence of high airway pressures and possible laryngospasm. A decision was taken to terminate the procedure immediately and she recovered with sequel and left hospital the same evening. This was reported as an Adverse Drug Reaction as such respiratory complications might occur after NAC infusion, although the attending anaesthetic team was not completely convinced this was due to NAC. The patient underwent LSG (outside of the study) 6 months afterwards without complication.

4.5.2.3 Adverse Events

As expected in a group of morbidly obese patients undergoing major abdominal surgery, there was a high rate of adverse events without any lasting clinical significance. Most patients had post-operative pain and nausea, along with transient episodes of tachycardia, pyrexia and signs of basal atelectasis. Although these were classified as adverse events for the purpose of trial reporting, they are not termed as such in this evaluation.

1 patient developed meralgia paraesthetica diagnosed following discharge. It is likely that compression of the lateral cutaneous nerves of the thigh occurred whilst recumbent during the initial post-operative recovery period. The patient required treatment with neuromodulating analgesia and recovered over the next few months. A full list of adverse events for each patient is given in Appendix **B1.6** (Table B.1).

Lengths of stay were similar, although 1 patient from each group did have prolonged readmissions within the reporting period, as detailed above.

Table 4.5 30 day Complications by Clavien-Dindo Classification (324)

Grade of Complication with explanation	Type of Complication	Control Group	NAC group
Grade 1: Any deviation from the normal postoperative course without the need for pharmacologic treatment or surgical, endoscopic, and radiologic interventions.	*total*	*2*	*0*
	Post-operative diarrhoea (no organism cultured)	2	0
Grade 2: Complications requiring pharmacologic treatment with drugs other than such allowed for grade 1 complications. Blood transfusions and total parenteral nutrition are also included.	*total*	*4*	*4*
	Reaction to NAC	0	1
	Blood transfusion	1	1
	Readmission for Poor oral intake	2	0
	Readmission for Hypotension	0	1
	Readmission for soft tissue infection in toe	0	1
	Meralgia Paraesthetica	0	1
Grade 3: Complications requiring surgical, endoscopic or radiologic intervention.	*total*	*0*	*2*
Grade 3a: Intervention not under general anaesthesia		0	0
Grade 3b: Intervention under general anaesthesia	Reoperation for Staple Line haemorrhage	0	1
	Reoperation for Leakage (and readmission)	0	1
Grade 4: Life-threatening complications (including central nervous system complications) requiring intensive care unit stay	*total*	*0*	*1*
Grade 4a: Single organ dysfunction (including dialysis)	Post-operative non-invasive respiratory support	0	1
Grade 4b: Multiorgan dysfunction			
Grade 5: Death of the patient		0	0

Qualitative make-up of each group was compared by Chi-square test. No significant differences between groups, although the numbers are too small to make a robust statistical conclusion.

4.5.3 Primary Clinical Outcome Measures

Baseline parameters, estimated Area under Curve (AUC) up to the fourth post-operative day and fold change (FC) values are given in Table 4.6. The values for each timepoint are presented in Appendix **B1.6** (Table B.2).

Table 4.6 Baseline values of parameters, AUC and Fold Change values in treatment and control groups

Parameter	Control (n=10)	NAC (n=9)
	Mean ± SEM	
Pre-operative values		
ALT (IU/L)	33 ±4	30 ±5
AST (IU/L)	28 ±2	26 ±3
WCC (x10^9/L)	8.6 ±0.7	10.3 ±1.3
CRP (mg/L)	15.6 ±3.1	17.5 ±4.8
PLT (x10^9/L	341 ±32	301 ±17
Area under curve		
AUC ALT	4058 ±1099	3034 ±662
AUC AST	2869 ±690	2125 ±350
AUC WCC	224 ±16	237 ±32
AUC CRP	1302 ±249	1513 ±399
AUC PLT	6060 ±595	5155 ±243
Fold changes		
ALT FC at 1hour	5.66 ±0.9	5.36 ±0.7
ALT FC at 24 hours	7.78 ±1.8	6.55 ±0.9
WCC FC at 1 hour	1.88 ±0.1	1.58 ±0.1
Peak CRP FC	11.59 ±2.8	9.04 ±2.4

The assumption of normality was assessed by Shapiro-Wilk's test ($p>0.05$). Values in each treatment group were compared using independent samples' t-test. Area under Curve (AUC) values from baseline to the fourth day were calculated using the trapezoidal rule. Fold change values were calculated as ratio of timepoint/baseline value. Fold changes were not normally distributed (Shapiro-Wilks test, $p<0.05$). Mann-Whitney-U test was used to compare treatment groups. All comparisons were statistically non-significant ($p>0.05$).

Normal ranges of values: Alanine aminotransferase (ALT) 5 – 55 IU/L; Aspartate Transaminase (AST) 10 – 50 IU/L; White Blood Cell Count (WCC) 4.0 – 11.0 x10^9/L; C-reactive protein (CRP) <5 mg/L; Platelet count (PLT) 150 – 450 x10^9/L

4.5.3.1 ALT

There were no significant differences at baseline (p=0.575). Following surgery, ALT rose, peaking at postoperative day 1. The rise in ALT at the end of surgery and on post-operative day 1 was significant (p<0.001). However there were no significant differences between treatment groups (see Figure 4.4). There was a wide variation between subjects in both groups in the extent of the ALT rise post-operatively and the standard deviations and confidence intervals are correspondingly large. AUC ALT was calculated to give a more general measure of the post-operative rise in ALT and values were not significantly different between treatment groups.

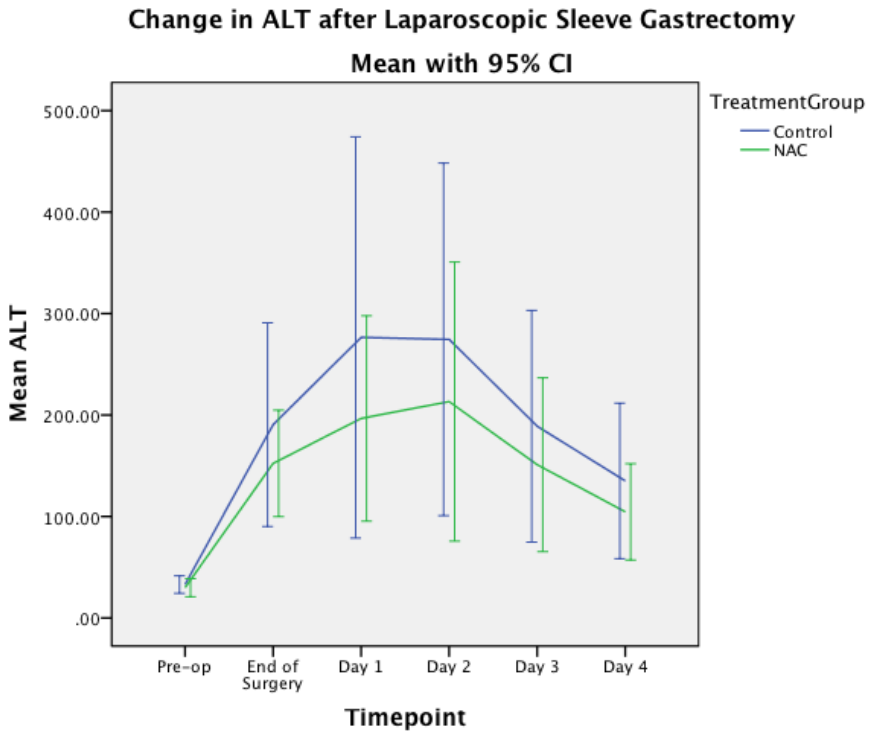


Figure 4.4 Change in post-operative ALT over first four post-operative days.
Values given as mean ALT (95% confidence intervals) IU/L. No significant differences between timepoints (p>0.05).

4.5.3.2 AST, WCC, Platelets, CRP

Data are also presented for changes in AST, WCC, CRP and Platelets (see Figure 4.5). Again, these demonstrate a lack of any significant differences between groups. There is a significant rise in AST and WCC between baseline, end of surgery and day 1 ($p<0.001$). CRP lags behind and rises significantly on days 1 and 2 ($p<0.001$), when it then reaches a plateau, before falling off more slowly. Platelet account appears to be depressed on day 1 and day2 from baseline ($p<0.01$).

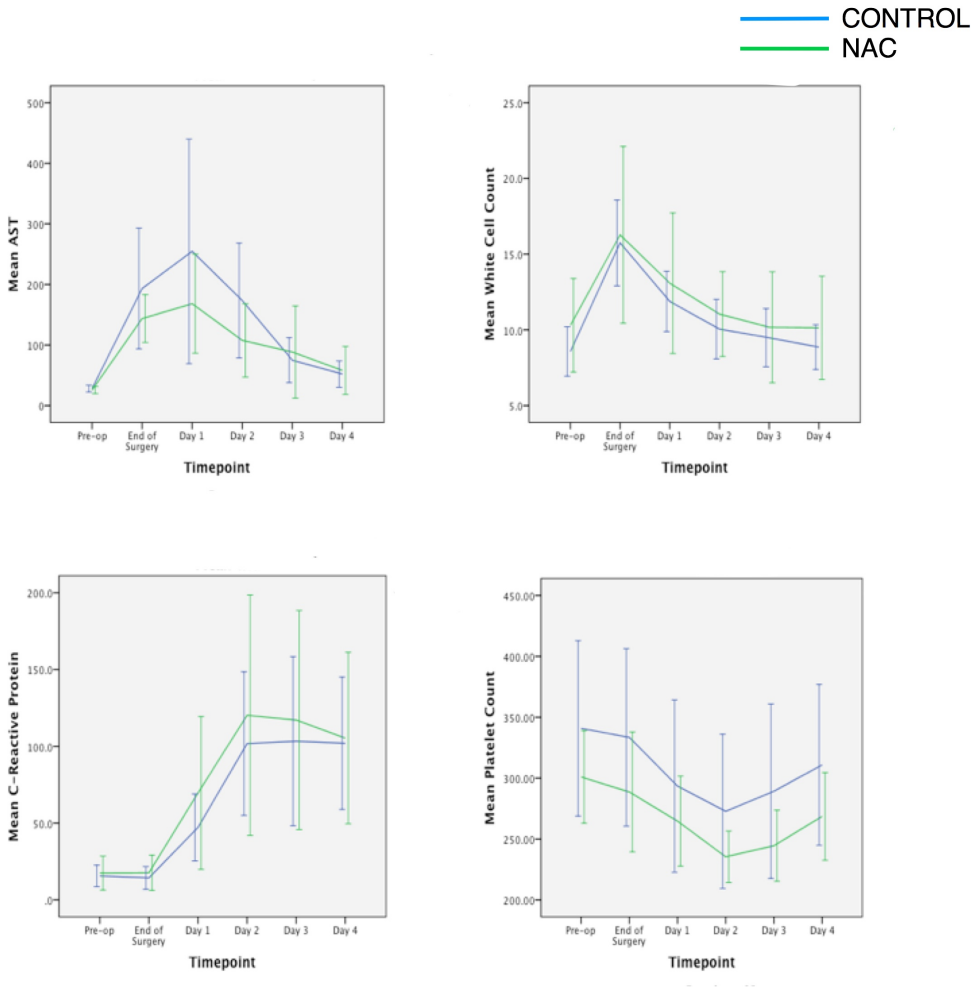


Figure 4.5 Changes in AST, WCC, CRP and Platelets over 4 post-operative days.
Values given as mean (95% confidence intervals). Units: AST IU/L, WCC $x10^9$/L, CRP mg/L, Platelets $x10^9$/L. No significant differences between timepoints ($p>0.05$).

4.5.4 Liver Histopathology Scores

Assessment of liver histology showed no significant differences between treatment groups, with only 5/19 patients having minimal signs of steatosis (see Table 4.7). The majority of patients had evidence of significant liver pathology. For a full list of diagnoses and liver injury scores, see Appendix **B1.6** (Table B.3).

Table 4.7 Histopathological diagnoses made from intraoperative liver biopsies

	Control (n=10)	NAC (n=9)
NASH	4	7
cirrhosis	1	
NASH with bridging fibrosis	1	
NAFLD	1	
normal/minimal steatosis	3	2

NASH – non-alcoholic steatohepatitis. All diagnoses made by expert liver histopathologist.

The mean difference in liver injury score (see Table 4.1 for criteria) between treatment groups of the start and end of surgery injury scores were not significantly different (mean difference in scores Control 0.50 ±SEM 0.52 versus NAC 1.56 ±0.58, p=0.19). In fact, the NAC group had numerically higher mean injury scores at the end of surgery compared to controls (see below Figure 4.6).

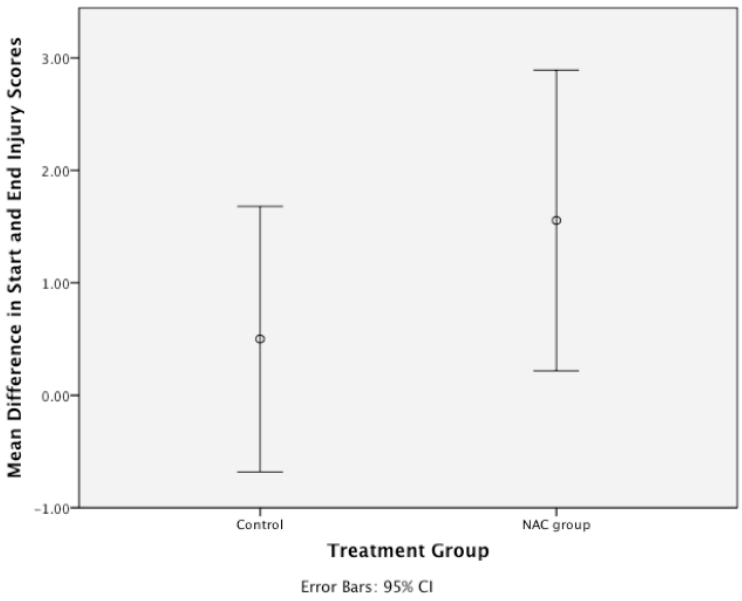


Figure 4.6 Mean difference (±95%CI) in liver injury scores between start and end of surgery (p>0.05).

Looking at the whole cohort of 19 patients together, the NAS did not change significantly between start and end of surgery (4.21 ±0.46 and 4.79 ±0.56, p>0.05). This is expected, as the components of NAS are chronic changes unlikely to be affected by any acute liver injury. The Injury Score did increase after surgery (2.21 ±0.22 to 3.21 ±0.39, p=0.022), although this was only by approximately 1 point (see Figure 4.7).

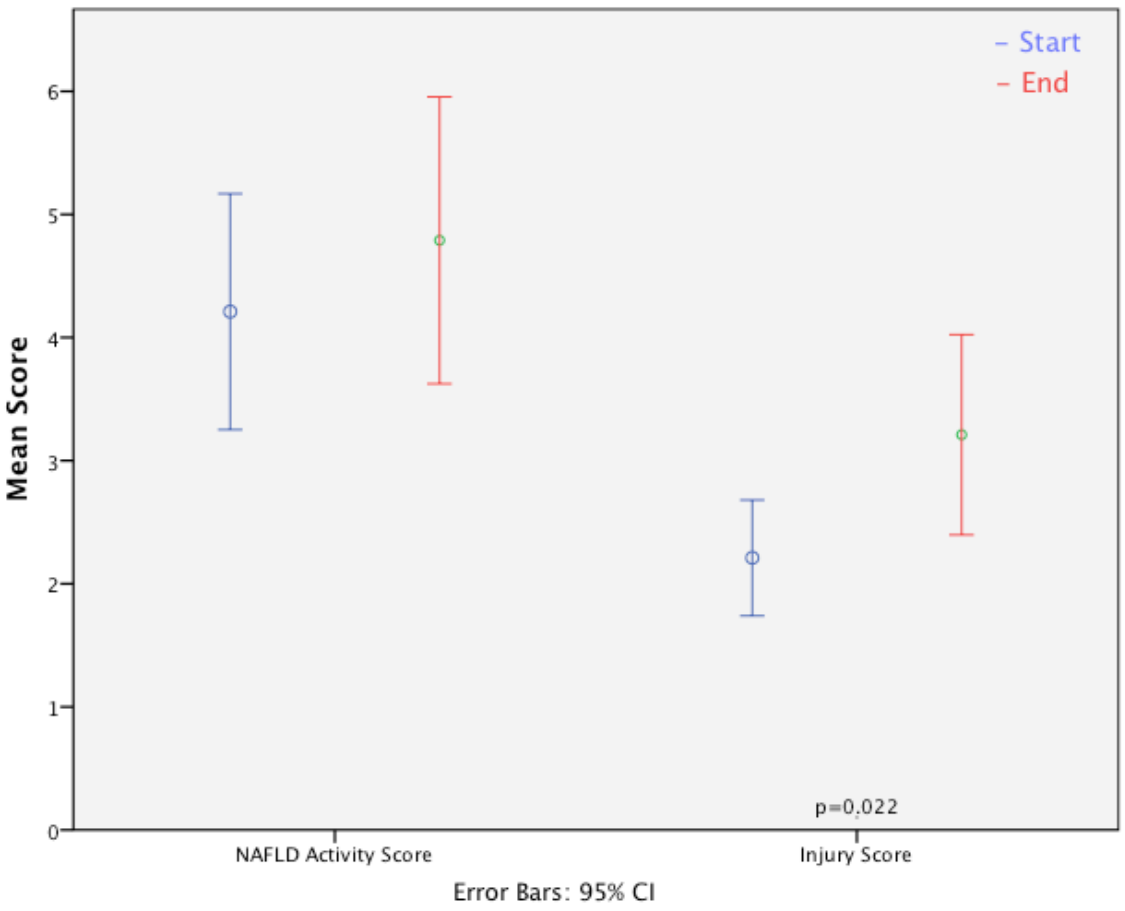


Figure 4.7 Mean NAFLD Activity Score (NAS) and Mean Liver Injury Score at start and end of surgery.

NAS ≥ 5 is associated with NASH. Mean injury score is significantly higher at end of surgery (p=0.022).

4.5.5 Correlations

Table 4.8 Clinical factors and outcome measures – Pearson's correlation r values

Parameter	AUC ALT	AUC AST	AUC WCC	AUC CRP	AUC PLT	Op time	LOS Days
BMI	0.441	0.269	-0.035	0.312	-0.44	-0.086	.551*
Blood Loss	0.063	0.122	.534*	.515*	0.323	.682**	-0.011
Liver retraction time	0.262	0.378	.499*	.463*	0.353	.841**	0.132
NAS at start	.637**	.586**	0.243	0.244	-0.194	-0.075	0.099
Fibrosis score at start	0.307	0.248	0.379	0.135	-0.135	-0.054	0.118
Difference in Start and End Injury Scores	0.301	0.442	-0.082	-0.069	-0.143	0.18	-0.205

AUC – area under curve, Op time – operation time, LOS – hospital stay
*p<0.05, **p<0.01.

NAS score at the start of operation strongly correlated with the AUC of ALT and AST (**p=0.003 and p=0.008 respectively). Blood loss and time of liver retraction correlated with WCC and CRP (*p<0.05). BMI correlated with post-operative length of stay. Operation time was strongly associated with longer liver retraction times and greater blood loss (p<0.001). Log10-operation time correlated strongly with log10-ALT 24 hour fold change (Pearson's r=0.556, p=0.017, see Figure 4.8 overleaf).

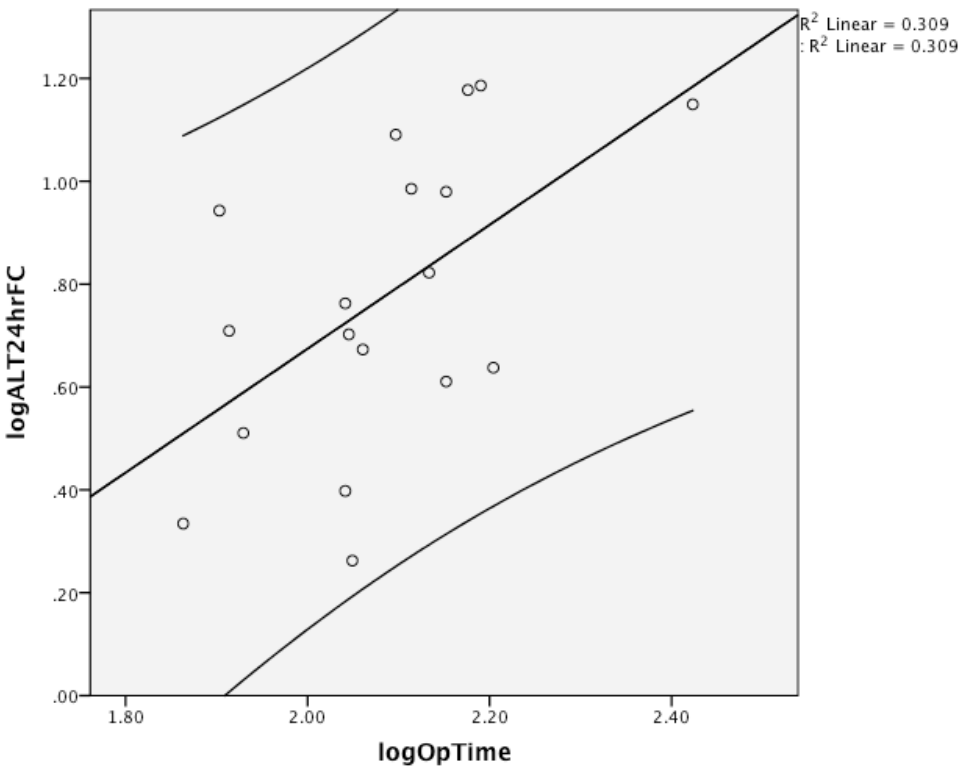


Figure 4.8 Scatterplot of Operation Time and ALT 24hour Fold Change after logarithmic transformation (base 10) showing significant association.
Curved lines are prediction intervals (Pearson's r= 0.055, p=0.017).

4.6 Discussion

4.6.1 The effect of NAC on liver function and liver histology

During laparoscopic bariatric surgery, the combination of pneumoperitoneum and mechanical liver retraction causes hepatocellular injury. These results demonstrate that by the end of surgery, a five-fold increase in ALT occurs. The injury peaks by the first post-operative day. The elevation is associated with a concomitant elevation in WCC, whilst CRP takes longer to rise, peaking by the second post-operative day. There is great patient-to-patient variation in the degree of post-operative elevation.

This is the first report of the use of NAC or any other antioxidant as a method of attenuating intra-operative liver injury in morbidly obese patients. The rationale for this study was based on evidence in our unit that NAC lead to improvement in measures of quality and metabolic function of hepatocytes isolated from steatotic livers. The administration of NAC in the perioperative period did not have a significant effect on post-operative parameters. There were no significant differences between treatment groups in ALT, AST, WCC, CRP and platelet counts.

The locally developed liver injury score was used to demonstrate that histological changes did occur during surgery, and the change in injury scores due to surgery was statistically significant, although the rise in score was only on average 1 point. Junnarkar *et al* used bucillamine in a rat model of liver ischaemia-reperfusion injury **(325)**. Buccilamine is also a thiol donor like NAC and acts as an antioxidant by increasing intracellular levels of glutathione. They employed the previously published Suzuki score of histopathological changes of necrosis, vacuolation and ballooning to assess liver injury **(326)**. We incorporated these features in a more comprehensive score, although congestion and vacuolation were the most important determinants of injury score in our study population. To the best of one's knowledge, there are no other reports of serial intraoperative liver biopsies in humans and these results. Although the left liver lobe looks discoloured and blueish after the removal of the liver retractor, these results demonstrate that histopathological changes at that point are limited. These results corroborate the findings by Alexakis *et al*, who found in their 11-parameter score that the majority

of individual scores were 0 or at most 1/3, with mean scores<1 **(232)**. These data show that histological changes due to pneumoperitoneum are subtle. Given that markers of liver injury and inflammation continue to rise on post-operative days 1 and 2, it is likely that further histopathological changes may become discernible at later timepoints. Of course, it would neither be safe or ethical to obtain liver tissue after the patient leaves the operating theatre in a clinical setting. Therefore, circulating markers of liver injury are more useful in this context.

NAS score correlated strongly with the AUC of ALT and AST, confirming that patients with higher grades of NAFLD/NASH are vulnerable to more hepatocellular damage **(181, 211)**. However operating and liver retraction times did not directly correlate with ALT or AST levels but there was a correlation between Log10 transformed operating time and the 24 hour ALT fold change, which is in keeping with other published findings **(27, 272)**. Liver retraction times correlated with AUC-CRP, indicating that inflammatory response is associated with liver injury.

4.6.2 Clinical outcomes of NAC Trial

The trial was not powered to detect differences in clinical outcomes. In general, a BMI >60kg/m^2 is associated with higher risk of complications following gastrointestinal surgery. Although the rate of life-threatening complications was low, there were a number of complications. One out of 19 (5%) patients had a staple line leakage. Published rates of leakage are between 1 and 7%, with a recent large series reporting a rate of 2.8% **(327)**. One other patient required a post-operative blood transfusion. The long staple line needed when resecting the stomach is a risk factor for both bleeding and leakage in LSG. Other recorded complications were not directly related to surgery itself. Readmission rate was high (4/20), and may reflect the high-risk nature of the super-obese cohort of study participants. Only one patient had a complication possibly related to NAC, and this complication was short-lived and self-limiting. NAC has been widely used in different patient populations and has a good safety profile. This is part of its attraction as a potential treatment.

The study groups were well matched, with similar demographic parameters, although there was a higher incidence of NASH in the NAC group. There was a high incidence of other obesity-related complications, especially diabetes, hypertension, hypercholesterolemia and obstructive sleep apnoea. Operating times and intraoperative blood loss were also not significantly different. Baseline outcome measures were also similar between groups, except for platelet count, which was non-significantly but persistently higher in the NAC group. Given that the difference was present at baseline, it is likely this is a chance finding.

4.6.3 Limitations of study

A major drawback of the study was a lack of a standardised, reproducible toxic insult to the liver during intraoperative liver retraction. The Nathanson liver retractor was placed during surgery to allow adequate visualisation of the hiatus for surgery to proceed safely. The pressure applied to liver varied from patient to patient depending on their body habitus, intraabdominal dimensions and size and texture of the liver. There are no routine clinical methods of measuring tissue oxygen tension or pressure within the liver. Some experimental methods do exist, including insertion of microdialysis catheters to allow continuous monitoring of metabolites, such as lactate, or of tissue pO_2 and pCO_2. It is also possible to use a surface oxygen saturation probe **(328)**, although this does not have approval for routine clinical use and would itself have required new MHRA(UK) approval for use as a medical device. Other authors have used laser Doppler imaging to measure end-organ perfusion but there are no approved devices for clinical use and the only published studies are in animal studies or isolated perfusion models. In terms of commonly used measures of liver perfusion, measurement of indocyanine green (ICG) clearance using a transcutaneous external probe allows determination of plasma disappearance rate of ICG and the retention rate of ICG after 15minutes **(329)**. These parameters estimate liver perfusion and liver excretory capacity, giving a functional measure of global liver function. There is one human study measuring liver perfusion at two different pneumoperitoneum pressures during laparoscopic cholecystectomy **(258)**. A 14mmHg pneumoperitoneum is associated with a statistically significant decrease in ICG plasma disappearance rate by the end of the operation. However, the significance

of this is questionable, and in contrast Hoekstra *et al* showed in a pig model that ICG clearance increases during pneumoperitoneum, suggesting a compensatory increase in global liver perfusion **(234)**. Therefore, it is open to question whether ICG clearance would be a useful method of quantifying liver ischaemia in the present study.

There were a number of other limitations in the methodology. The trial design was deliberately pragmatic and therefore patients were not stratified by their other co-morbidities and allowed to continue their regular medications. Eleven (58%) of patients completing the study were taking cholesterol-lowering medications, including various statins and fenofibrate. These drugs are known to cause liver dysfunction **(330)**, although it should be noted that all patients had normal liver function at baseline. Similarly inhalational anaesthesia may have an effect on post-operative liver function, due to their metabolism in the liver and their effect on hepatic blood flow **(331)**. The dosage of anaesthetic drugs used varies from patient to patient and between anaesthetists, creating another confounding variable. Conversely, sevofluorane may offer a protective effect, as a pharmacological postconditioning agent as demonstrated in a randomised trial of patients undergoing liver resection **(332)**. Beyaz *et al* investigated the use of NAC in patients undergoing laparoscopic gynaecological surgery under isofluorane anaesthesia. Contrary to their rather misleading conclusions, they noted no significant differences in post-operative liver function tests between groups and indeed both groups had lower ALT and AST levels after surgery compared with baseline. Simvastatin may protect the liver and suppress caspase-mediated apoptosis **(333)**. The study participants were all in the super-morbidly obese category and at greater risk of peripheral skeletal muscle injury, which may also contribute to some of the transaminase rises although no patients had clinical evidence of rhabdomyolysis **(334)**. Post-operative oral administration of methylene blue, as a method of diagnosing a staple line leak, may also have affected the results as methylene blue has well known anti-oxidant effects **(335)**.

In this study, the timing of the injury to the liver was intraoperative. Taking the results presented here which show peak transaminase rise on post-operative day 1 together with other evidence from the ischaemia-reperfusion injury literature, it is

likely that the effects of liver retraction, due to compression and ischaemia, might persist for up to 12 and perhaps more than 24 hours after surgery **(179)**. It could be argued that a one-off infusion of NAC is insufficient to counteract this injury. Many animal models investigating NAC and IRI, infusion continued for more than 6 hours **(309)**. In other trials investigating NAC in an acute setting, infusion was continued for much longer: 24 hours after aortic aneurysm surgery **(336)**, 48 hours for critical care patients who were hypotensive **(337)** and for 7 days in patients with severe acute pancreatitis **(294)** and acute liver failure **(338)**. The volume of carbon dioxide gas or the actual measured intraoperative pneumoperitoneum pressures (as opposed to merely the setting on the insufflator machine) were not recorded in this study and variations in flow and pressure may have differentially affected hepatic blood flow **(339)**. Measurement of intraoperative and post-operative lactate levels may have been a useful marker of overall acid-base balance and end-organ tissue perfusion .

4.6.4 Potential sources of bias in this trial

The method of allocation to study groups, using sealed envelopes and block randomisation, can be criticised and presents a higher risk of bias than other potential methods **(340)**. The difficulties of minimising bias in surgical trials is well known **(341)**. It was not possible to use a third party for control of randomisation due to lack of funds. Nevertheless, the primary outcome measures are objective laboratory assays that are at low risk of bias. In this study, it was not feasible to use a placebo infusion for NAC due to lack of access to such an infusion. More importantly, NAC is administered after dilution in 1 litre of 5% dextrose solution and a plain dextrose infusion was not given to the control group. This may have been another confounding factor. Dandona *et al* found that glucose infusion in patients with diabetes induced oxidative stress within 6 hours of administration **(342)**.

The most important factor in the interpretation of the results is the small sample size and premature closure of the trial. The trial was stopped early after a planned interim analysis due to a number of factors. Rate of recruitment over the two years was low. Following initiation of the trial, other studies in the surgical unit

were recruiting patients specifically for Roux-en-Y gastric bypass. Laparoscopic AGB was increasingly performed as day-case procedures, which meant that potential participants could not complete the other study-specific interventions. This meant these two groups of patients were no longer available for recruitment. At the ongoing rate of accrual to the trial, it may have taken many more years to reach the stipulated sample size.

Analysis of the primary outcome measures, especially ALT and AST, along with the cytokines and markers of oxidative stress, showed a large variation in values. This is likely to be multi-factorial. Reasons for the variation include the variability of the degree of compression and ischaemia induced by liver retraction, the groups were not stratified by liver pathology or other co-morbidities and the inherent unpredictability in the variation of the inflammatory insult for each patient. For example, Nguyen *et al* also reported very large variation in the peak post-operative ALT after RYGB (mean 152 SD± 102 U/L) **(229)**. In a larger sample size, the bias introduced by this variability would have been offset by the randomisation to each study group. However, taking into account the other issues discussed, the opinion of the trial steering committee was that these limitations could not be overcome within the framework of the existing trial design and that further recruitment would likely be futile. As a result of early stoppage, the study is underpowered and there is a risk that this negative result is a Type II statistical error (inferring NAC was ineffective in reducing hepatocellular injury when it is in fact effective). Given that there were more Grade 3 complications in the NAC group, it may also be possible that administration of NAC lead to worse clinical outcomes. Again, a larger trial would have addressed this question more robustly.

4.7 Conclusions

This study has demonstrated that it is feasible and safe to include super-obese patients in surgical trials. To the best of one's knowledge, there are no other reported surgical trials in patients with a mean BMI >60 kg/m^2. The main conclusion is that NAC does not ameliorate liver damage, as quantified by ALT and AST, following laparoscopic bariatric surgery. The results demonstrate that significant hepatocellular injury, as measured by ALT and AST, occurs within 24 hours of surgery. This is accompanied by an inflammatory response, characterised by a rapid rise in WCC by the end of surgery, and a delayed rise in CRP, peaking on the second post-operative day. A comprehensive liver injury score is able to quantify objectively liver damage, although such changes are still minor at the end of the operation. Rates of complications after surgery in both treatment groups were significant, although the trial was not sufficiently powered to reach conclusions about the efficacy of NAC on clinical outcomes. The study design could be improved if the intraoperative insult to the liver could be accurately quantified. Larger studies, with stratification of patients according to their co-morbidiities, would lead to more robust conclusions about the efficacy of NAC in this setting.

CHAPTER 5:

ACUTE CHANGES IN OXIDATIVE STRESS, CYTOKINES AND HEPATOCYTE DEATH AFTER LAPAROSCOPIC BARIATRIC SURGERY

5.1 Introduction

The pathophysiologies of obesity-induced inflammation and oxidative stress have been discussed in Chapter 1 and 2, along with the particular concerns about fatty liver disease. Chapter 3 covers the pathophysiological effects of pneumoperitoneum and the potential effects of liver retraction in triggering an ischaemia-reperfusion injury. The clinical outcomes of the NAC trial are detailed in Chapter 4. This chapter includes the secondary non-clinical measures evaluated in the context of the NAC trial. More detail is given of the particular cytokines, biomarkers and other assays used in evaluating the inflammatory and oxidative stress response after surgery. These markers have been discussed previously in the context of the various pathophysiological mechanisms of disease previously. The laboratory methods described in this chapter will also pertain to the longitudinal studies described in subsequent chapters.

5.2 Background

5.2.1 Assessment of liver injury

Ischaemia and ischaemia-reperfusion injury mediate their effects through the production of reactive oxygen species, leading to direct cellular damage, oxidative stress and production of pro-inflammatory stimuli **(343)**. In turn, this can trigger the systemic inflammatory response. Hepatocellular injury leads to a local response as well, creating a pro-inflammatory, pro-fibrogenic environment (see Figure 5.1). Both systemic and local changes can be detected in the serum **(344)**, using biomarkers for inflammation (including interleukins 6 and 10, tumour necrosis factor alpha, C-reactive protein), fibrosis (for example, hyaluronic acid), oxidative stress (including lipid peroxidation status, glutathione enzymes) and apoptosis (for example cytokeratin 18, Fas ligand). The expression of genes within

the liver controlling these processes of inflammation, fibrosis, oxidative stress and apoptosis can be analysed using molecular biology techniques, including quantitative real-time polymerase chain reaction and microarray technology **(345)**.

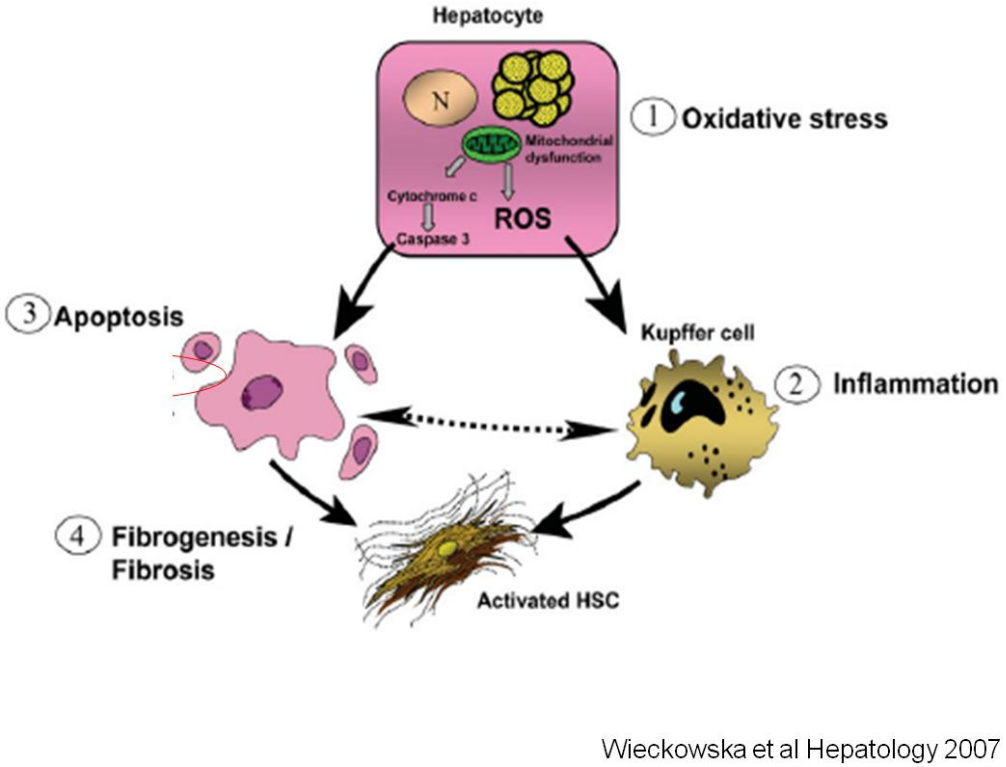


Figure 5.1 Summary of the cellular processes involved in liver injury that leads to non-alcoholic steatohepatitis (adapted from Wieckowska *et al*, 2007) (344).
Hepatocyte steatosis is a prerequisite for the subsequent events that lead to liver injury and fibrosis.

Obviously, it would be impractical (and unaffordable) to measure all potential markers used in the literature. When designing these studies, a range of markers has been selected to exemplify and account for the main processes in question: inflammation, oxidative stress and hepatocyte apoptosis. Fibrogenesis is a more chronic process and hyaluronic acid has been measured over longer timepoints, and this is described in Chapter 6. It was also not possible to perform comprehensive molecular biology studies of the samples taken in the NAC trial as part of this thesis but a 'proteomic-style' approach was adopted in measuring a range of apoptosis-related proteins, as described below.

5.2.2 Obesity-related inflammation

The common inflammatory processes implicated in the pathogenesis of insulin resistance, obesity and fatty liver disease are also of great relevance to the pathological effects of ischaemia-reperfusion injury (IRI). Excessive calorie intake leads to accumulation of fat and an increase in adipocyte secretion of proinflammatory cytokines, coupled with a decrease in anti-inflammatory mediators (as depicted in Figure 5.2). A similar change in balance occurs within fatty liver **(346)**. Over 30 cytokines have been identified as playing a role in obesity-related inflammation (see Table 1.4, page 33). This is not an exhaustive description and the exact interplay between all of the various mediators *in vivo* is yet to be fully elucidated **(346)**.

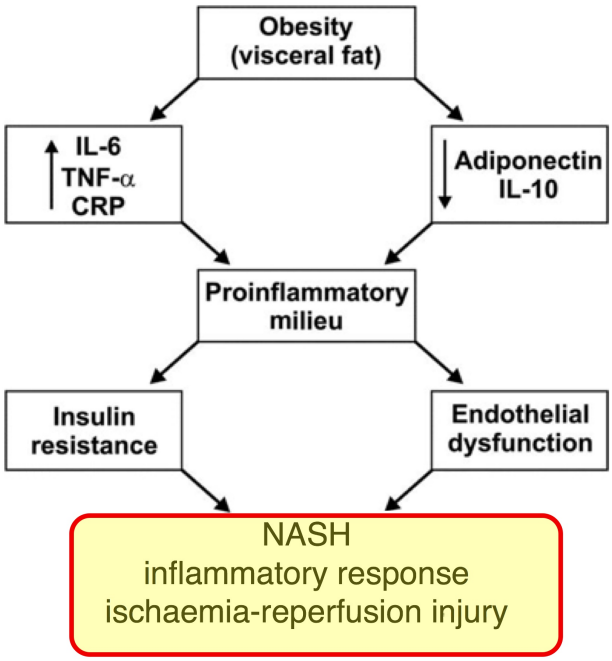


Figure 5.2 The common factors in morbidly obese patients cause short-term and chronic problems (adapted from Esposito *et al*, 2006) (347)

5.2.3 Cytokines

5.2.3.1 TNFα

This is the classic obesity associated pro-inflammatory cytokine and was first associated with obesity and insulin resistance more than 20 years ago **(348)**. Evidence for its effects comes from various knockout mouse models and cross-sectional clinical studies of obese subjects **(346)**. TNFα is present in low

concentrations in normal weight subjects and is increased in both T2DM and NAFLD **(139)**. It is secreted by macrophages and other immune cells within the adipose tissue and liver **(90)**. Its mechanism of action is through two TNFα – receptors. Three different intracellular pathways are associated with TNFα **(349)**. The chronic metabolic effects are effected through phosphorylation of mitogen-activated protein kinases. A series of further processes leads to phosphorylation of the insulin receptor, leading to insulin resistance. Activation of nuclear factor-κβ (NF-κβ), a nuclear transcription factor, leads to phosphorylation and transcription of a number of nuclear proteins involved in cell survival and proliferation, inflammatory response and anti-apoptosis mechanisms. Finally, TNFα has a role in death receptor activation, activating caspase-mediated apoptosis. The balance of the various pathways and effects is not fully understood but can be affected by levels of other cytokines and ROS. Clinically, its effects include stimulating fever, promoting chemoattraction to neutrophils, increasing neutrophil migration into tissue by increasing expression of adhesion molecules on endothelial cells, stimulating macrophage phagocytosis, and increasing liver production of CRP and other acute phase response proteins **(349)**. TNFα expression is stimulated by toxic and inflammatory stimuli, including bacterial endotoxins, and other cytokines.

5.2.3.2 Interleukin 6

IL-6 is another pro-inflammatory cytokine involved in the immune response and implicated in the obesity-related pathogenesis of insulin resistance and NAFLD, as well as other auto-immune and chronic inflammatory diseases **(346)**. It is released by immune cells and increased levels in adipose tissue are found in obesity **(347)**. It is an important mediator of the inflammatory response, and stimulates fever and production of CRP by the liver. CRP is involved in humoral immunity, binding to dead cells and bacteria and activating the complement system **(90)**. Elevated IL-6 is associated with a number of chronic inflammatory and auto-immune diseases, including systemic lupus, rheumatoid arthritis, chronic lung disease, Castleman's disease and inflammatory bowel diseases **(350)**.

5.2.3.3 Interleukin 10

IL-10 is traditionally classed as an anti-inflammatory cytokine, although it is more accurately described as an immunoregulator as it can stimulate some aspects of the inflammatory response **(351)**. Low IL-10 levels are associated with obesity, insulin-resistance and hepatic steatosis **(348)**. Exogenous IL-10 administration and knockout models suggest that inhibition of IL-10 production plays an important role in development of NAFLD and insulin resistance **(352)**. It is released by macrophages and other immune cells in response to bacterial toxins, catecholamine release and pro-inflammatory cytokines, including TNFα **(352)**. A NF-κβ pathway controls transcription and IL-10 itself has a negative feedback effect on the expression of other pro-inflammatory cytokines. The anti-inflammatory hypothesis was supported by IL-10 knockout mice models that had lethal sequelae of increased inflammatory bowel pathology, and down-regulation of colitis by administration of exogenous IL-10 **(353)**. Low IL-10 levels are associated with metabolic syndrome and obesity **(347)**. IL-10 expression on macrophages in adipose tissue was increased after RYGB-induced weight loss and this was associated with a decrease in pro-inflammatory cytokine and cell surface marker expression **(93)**. The authors of this study postulate that changes in IL-10 in adipose tissue reduce macrophage infiltration, which is thought to be a key event in creating a pro-inflammatory state in obesity. However, there is increasingly contradictory evidence about the true role of IL-10, and it may be simplistic to deem it a purely anti-inflammatory cytokine. Elevated IL-10 is associated with lymphoma **(352)** and a poorer prognosis after acute coronary syndrome **(354)**.

5.2.4 Adipocytokines

Collectively more than 50 cytokines have been isolated from adipose tissue and are implicated in obesity related diseases **(355)**. The cytokines that are predominantly or only secreted by adipose cells have been termed the adipokines. Leptin and adiponectin are probably the best known and most widely researched and there is increasing interest in the role of resistin in development of insulin resistance. I will discuss these in detail. There has been increasing interest in

adipocytokines in other acute settings **(356)**. Adipocytokines may be useful in predicting severity and need for intervention in acute pancreatitis **(357)**.

5.2.4.1 Leptin

Leptin was initially seen as a modulator of energy homeostasis and thought to exert its effects mainly as an endocrine signal **(358)**. Leptin levels are proportional to adipose tissue mass and therefore very high in the morbidly obese. Lately its role in the immune response is increasingly recognised, in the setting of acute sepsis and in chronic inflammatory diseases **(359)**. Leptin is increased by other inflammatory stimuli, including pro-inflammatory cytokines like IL-6 and TNFα and levels fall after weight loss. As well as centrally regulating appetite and energy homeostasis, leptin has peripheral effects on the immune system, including macrophage activation, neutrophil chemotaxis and modulation of T-helper cell differentiation. Bariatric surgery is associated with dramatic falls in leptin, potentially because of loss of fat depots following weight loss **(59, 87, 101)**.

5.2.4.2 Adiponectin

Adiponectin changes negatively correlate with weight loss and are associated with improved insulin sensitivity **(346)**. Its secretion by adipocytes is inhibited by TNFα and IL-6 **(355)**. It acts on the liver to increase fatty acid oxidation and reduce gluconeogenesis and exogenous adiponectin may stimulate lipid clearance from steatotic liver **(195)**. Adiponectin appears to exert opposite effects on immune cells to pro-inflammatory mediators, reducing cell adhesion molecule expression, inhibiting production of TNFα and inactivation of NF-κβ pathways. There is much interest in its anti-atherogenic effects and low adiponectin levels are associated with vascular complications of diabetes and the metabolic syndrome **(360)**. Bariatric surgery is associated with an increase in circulating adiponectin levels **(361)**.

5.2.4.3 Resistin

Resistin has been isolated from adipocytes, macrophages and its expression is increased in experimental models of asthma and inflammatory bowel disease **(355)**. Resistin levels correlate positively with BMI and adipose tissue mass and insulin resistance, although there are many conflicting reports of the exact nature of this association **(362)**. Resistin is also associated with a pro-inflammatory response, inducing TNFα and IL-6 secretion by activation of NF-κβ in mononuclear cells **(355)**. High resistin mRNA expression with liver tissue correlates with increasing NAFLD activity score (NAS) **(363)**. The effect of bariatric surgery on resistin levels is not yet clear, with conflicting results **(364)**. Post-prandial resistin levels are higher after LSG than RYGB, suggesting that its release may be mediated by gut hormones and affected by bypassing the proximal small bowel **(365)**.

5.2.5 Oxidative Stress Markers

The concept of oxidative stress has been described earlier and refers to an overload of cellular antioxidant mechanisms so that ROS-mediated tissue injury can occur. Quantification of oxidative stress is directed at measurement of known antioxidant compounds, such as vitamins E and C, assessment of antioxidant enzymatic activity such as SOD and GPX, and detection of byproducts of tissue injury, caused by lipid peroxidation, protein oxidation and DNA oxidation (summarised in Figure 5.3) **(366)**. Oxidative stress markers are by their nature often unstable and prone to producing artefactual results due to inconsistencies in sample procurement, storage and processing. Thus, there are significant variation between studies and no standardised reference ranges have been established, in contrast to routine clinical parameters, such as renal and liver function tests and full blood count indices **(272)**. In general, determination of oxidative stress markers using more complex methods, such as high-pressure liquid chromatography (HPLC) or mass spectrometry (MS), is more accurate than enzymatic methods. Some enzyme-linked immunosorbent assays (ELISA) have been developed but in general, the byproducts are small molecules that do not elicit specific antibody production **(366)**.

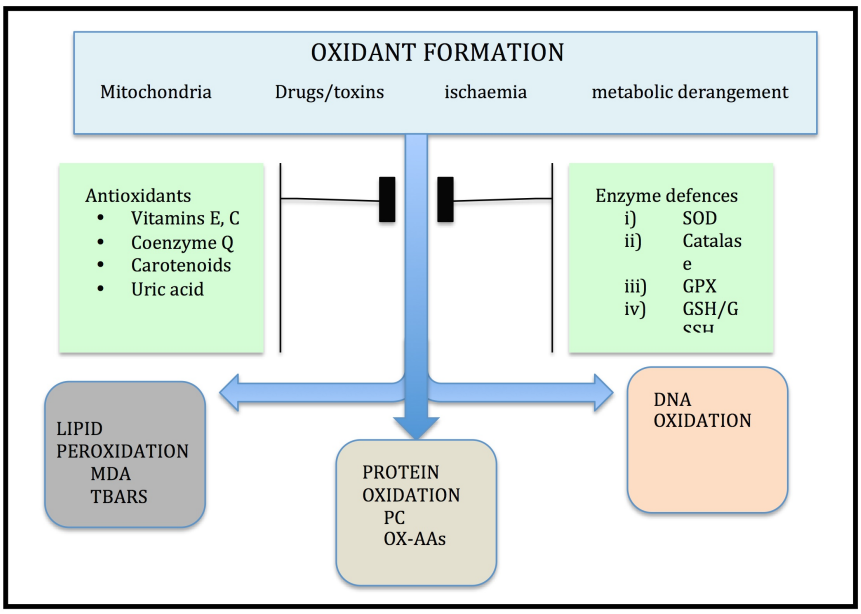


Figure 5.3 Summary of the effects of oxidative stress and potential methods of measurement (adapted from Grune and Berger, 2007) (366)

Taking into account feasibility and availability in the unit's laboratories, the following assays to measure oxidative stress in this study: thiobarbituric acid reactant substances (TBARS) as a measure of lipid peroxidation; protein carbonyls as a measure of protein oxidation; SOD and GPX, both antioxidant enzymes.

5.2.5.1 TBARS

Following the production of excess reactive oxygen species and other free radicals due to oxidative stress, enzymatic damage to cell constituents, including lipids and protein occurs. Enzymes such as myeloperoxidase produce toxic acidic compounds including hypochlorous acid, leading to peroxidation of lipids. Measuring lipid hydroperoxides directly is technically difficult. The TBARS assay quantifies the concentration of malondialdehyde (MDA), the most abundant product of lipid peroxidase, by way of an enzymatic reaction with thiobarbituric acid (TBA), producing a fluorescent product that can be measured with a spectrophotometer. MDA is not the sole product of lipid peroxidation and the TBARS assay is merely an approximation of a much more complex process. Nevertheless, it remains one of the most widely used measures of oxidative stress. The assay is standardised and reproducible. A number of studies have demonstrated increasing TBARS/MDA levels after laparoscopic surgery **(272)**. Bariatric surgery is associated with significant reductions in lipid peroxidation, as a consequence of weight loss and improved glycaemic control **(108, 110)**.

5.2.5.2 Superoxide Dismutase

Superoxide Dismutase (SOD) is a metalloprotein-based family of enzymes found in all cells. SOD catalyses the (dismutation) reaction of potentially toxic superoxide anion radicals (O_2^-) into less dangerous oxygen and hydrogen peroxide, which itself is broken down further. Both of these ROS can cause lipid and protein damage **(367)**. SOD is mainly present in the cytosol and its expression is induced by oxidative stress and pro-inflammatory cytokines, such as interleukin-1 and TNFα **(368)**. An increase in SOD levels is a protective mechanism and there is much interest in therapeutic inducement of SOD expression by plant extracts **(368)**. Mutations in SOD genes are associated with genetic diseases, such as motor neuron disease, and reduction in SOD activity is associated with development of various chronic diseases, including asthma and hypertension **(367)**. Bariatric surgery is associated with a reduction in SOD after 6-12 months, which is taken as a beneficial indicator of reduced oxidative stress **(110)**. In a cross-sectional study of oxidative stress markers after cholecystectomy, SOD activity was higher at the end of laparoscopic surgery than in non-operated controls **(369)**.

5.2.5.3 Protein Carbonyls

The production of reactive oxygen and nitrogen species during periods of oxidative stress can lead to damage to cellular proteins. These reactions often involve the oxidation of protein side chains, forming aldehydes and ketones. These are together referred to as carbonyls, owing to the Carbon-Oxygen groups on these side chains. Protein Carbonyls (PC) are very stable and therefore provide a good global measure of oxidative stress. In an acute setting, PC are increased following laparoscopic cholecystectomy, with greater pneumoperitoneum pressures of 15mmHg associated with larger rises in PC levels **(370)**. Morbidly obese patients have elevated PC compared with normal weight controls and fell by approximately 30% 6months after weight loss surgery **(371, 372)**.

5.2.5.4 Glutathione Peroxidase

Glutathione Peroxidases (GPX) are a family of cytosolic enzymes which act as scavengers of hydrogen peroxide, converting H2O2 to water by reducing oxidised

glutathione. Hydrogen Peroxide is a by-product of SOD and is produced after dismutation of reactive oxygen species, which are generated by oxidative stress. GPX-1 expression in the livers of obese patients undergoing bariatric surgery correlates with the severity of NAFLD Activity Score (NAS) **(373)**.

5.2.6 Apoptosis

Apoptosis is the term given to programmed cell death and is an energy-dependent process, whereby cellular constituents are broken down by caspases into smaller fragments **(374)**. These apoptotic bodies can then be phagocytosed. In healthy tissue, the process of apoptosis is a controlled and necessary process, to dispose of unwanted cells without exciting a massive inflammatory response. In contrast, cellular necrosis is the result of acute injury and results in the uncontrolled release of cellular components, which may excite an inflammatory response and immune cell reaction. In the setting of liver disease, apoptosis occurs in various chronic and acute states, including fatty liver disease and ischaemia-reperfusion injury, although this is not a binary process, but rather a spectrum involving varying combinations **(375)**.

Apoptosis in the liver may occur via three main pathways: the extrinsic or death receptor pathway, which involves activation of caspases after ligand-death receptor binding occurs; the intrinsic pathway, where intracellular activation of the caspases occurs due to damage to cellular components, including DNA fragmentation, leading to mitochondrial dysfunction; and the lysosomal pathway, where toxic damage or death receptor binding leads to release of enzymes capable of breaking down intracellular proteins **(290)**. Both intrinsic and lysosomal pathways involve changes in transcription of the pro- and anti-apoptotic factors (for example, *bcl-2, bax, bid, bim*). Thus, the process of apoptosis may be detected by the presence of pathognomic cellular fragments, including caspase-cleaved cytokeratin-18 (CK-18) fragments, or by measuring the relative expression of pro- and anti-apoptotic genes **(376)**. In contrast, there are no specific circulating markers of necrosis, although CK-18 M65 is used as a measure of total hepatocellular death. In Figure 5.4, the huge number and complexity of the various proteins, enzymes and genes involved in apoptosis are depicted **(377)**. It is beyond the scope of this thesis to discuss this in more detail.

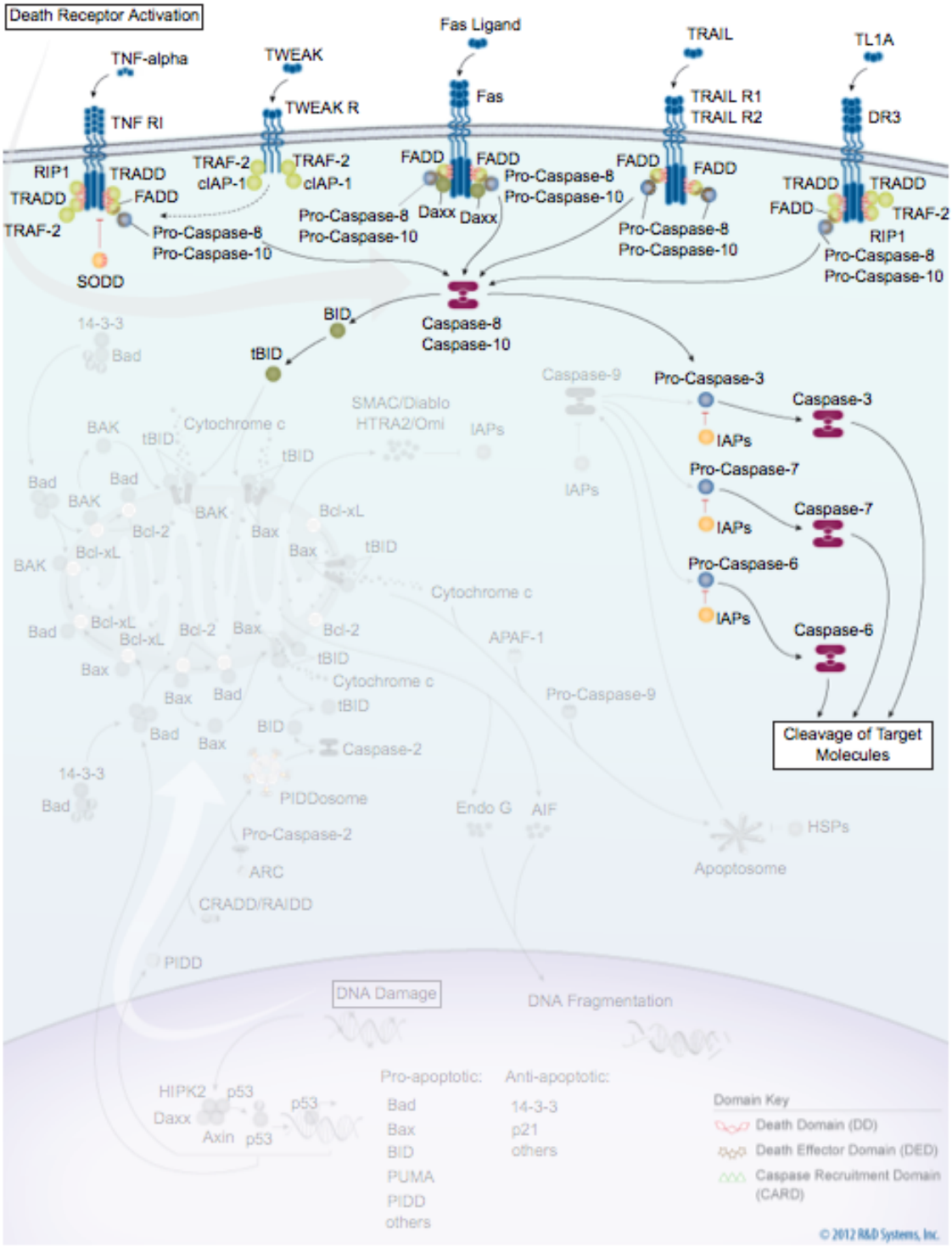


Figure 5.4 (a) Extrinsic and (b) Intrinsic Pathways of Apoptosis (reproduced from R & D Systems website) (377)
http://www.rndsystems.com/Pathway.aspx?p=15445&r=15436

The various genes and proteins expressed in the apoptosis pathway. TRADD - Tumour necrosis factor receptor type 1-associated DEATH domain, TWEAK - TNF Related Weak Inducer Of Apoptosis, FADD - Fas-Associated protein with Death Domain, IAP - Inhibitors of Apoptosis

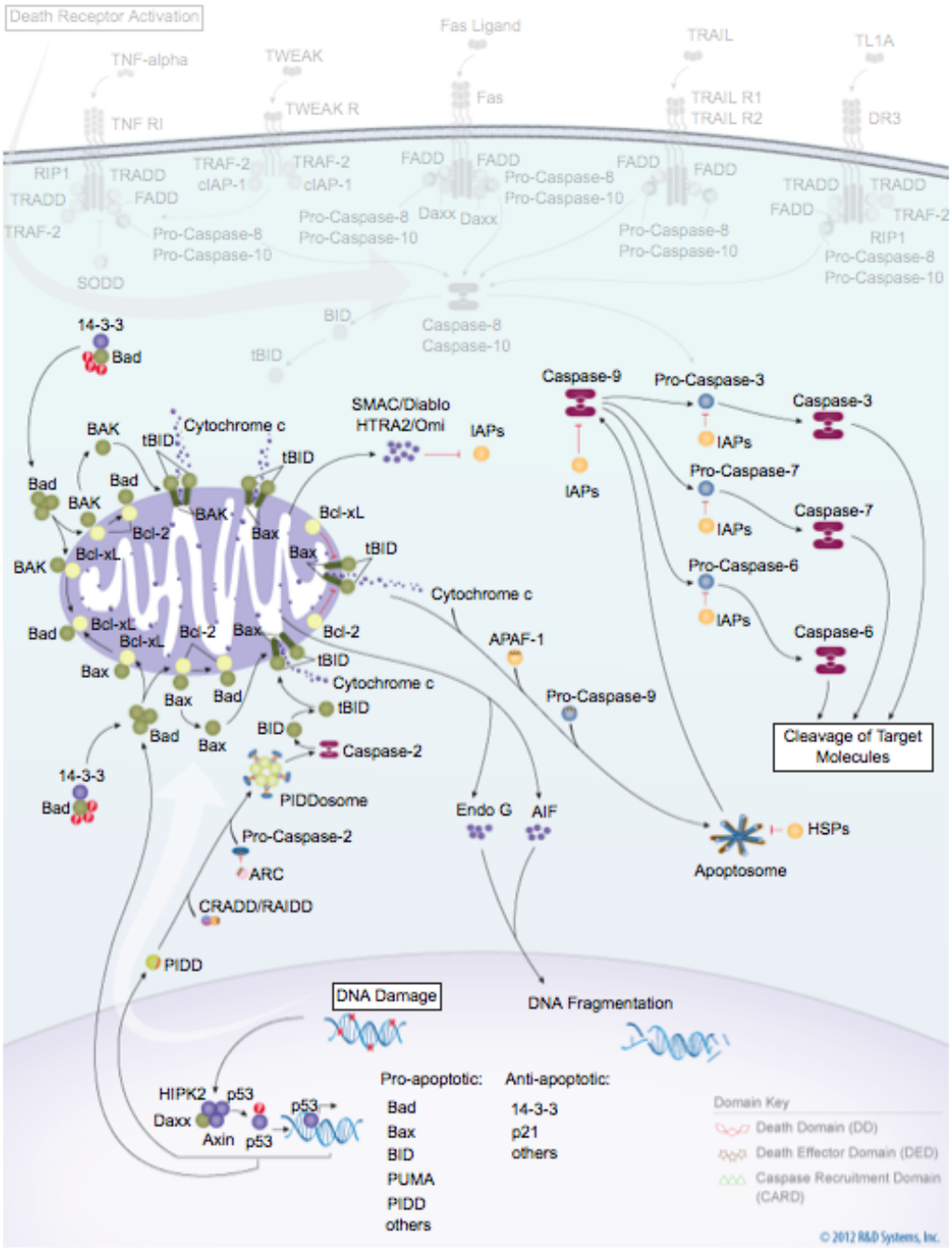


Figure 5.4(b) Intrinsic Pathways of Apoptosis (reproduced from R & D Systems website) (377)
http://www.rndsystems.com/Pathway.aspx?p=15445&r=15436

The various genes and proteins expressed in the apoptosis pathway. TRADD - Tumour necrosis factor receptor type 1-associated DEATH domain, TWEAK - TNF Related Weak Inducer Of Apoptosis, FADD - Fas-Associated protein with Death Domain, IAP - Inhibitors of Apoptosis

5.2.6.1 Cytokeratin 18 fragments

CK-18 is an intracellular protein expressed in high levels in cells of epithelial origin. Fragments of CK-18 are released into the circulation following cell death and can be used as a biomarker. CK-18 is cleaved by caspases into smaller fragments during apoptosis. CK-18 M65 Assay measures the total level of CK-18 in the serum, giving a measure of total cell death, by any cause, including necrosis or apoptosis. CK-18 M30 is a caspase-cleaved fragment and the assay allows us to measure the extent of cell death by apoptosis. Combination of M65 and M30 Assay results allows a determination of the approximate contribution of apoptosis in measuring cell death, although precise quantification of this contribution may not be possible. Cytokeratin-18 (CK-18) has been validated for use as a non-invasive diagnostic marker of NASH/NAFLD **(175)**, although its clinical utilisation is still not widespread. There is also much interest in its use as a tumour marker for various cancers **(378)** and as a prognostic indicator in sepsis **(379)**. In liver disease, there is increasing recognition of its value as a marker of severity and survival in acute liver failure **(380)** and paracetamol-induced liver injury **(381)**.

5.2.6.2 TRAIL

Tumour Necrosis Factor- Related Apoptosis-Inducing Ligand (TRAIL) is a widely expressed protein that is thought to induce apoptosis in a variety of cells. There is much interest in its role in liver diseases. Changes in the balance between TRAIL and various TRAIL decoy receptor ligands are thought to affect the extent to which TRAIL induces apoptosis. TRAIL-TRAIL Receptor binding is an important upstream part of the apoptosis pathway leading to caspase-induced protein cleavage. Having established with the CK-18 M30 assay that a degree of apoptosis-induced cell damage occurs after surgery, we have assayed TRAIL concentration in serum to ascertain possible modes of apoptosis. . TRAIL has been implicated in the development of hepatic steatosis **(382)** and steatotic hepatocytes are more susceptible to TRAIL-mediated apoptosis **(383, 384)**.

5.2.6.3 Fas Ligand

FasLigand (FasL) is a member of the tumour necrosis factor superfamily of cellular proteins, and is involved in the regulation of apoptosis. Binding to Fas leads to the activation of the Fas-associated death domain and caspase-8, and further downstream activation of caspases leading to apoptosis. Circulating FasL concentration may therefore be a marker of the mode by which hepatocellular apoptosis occurs. Elevations in FasL are seen after liver transplantation and blockage of FasL/Fas receptor interaction reduces apoptosis in a rat model **(385, 386)**

5.3 Aims and Outcomes

Following on from the clinical results of the NAC trial detailed in Chapter 4, the aim of this study is to determine if administration of NAC reduces post-operative oxidative stress, inflammatory mediators and biomarkers of liver injury after bariatric surgery. By understanding the changes in these markers, it may be possible to elucidate the main mechanism of action of NAC in this clinical setting. These results are the first time this combination and breadth of biomarkers have been studied in the first days after bariatric surgery.

The outcome measures used in this study are:

- circulating biomarkers of inflammation, fibrosis, liver apoptosis and oxidative stress
- changes in the expression of apoptosis related proteins in the liver

5.4 Methodology

5.4.1 Patients and Interventions

Patients were recruited as part of the NAC Trial. Protocol details are given in **Section 4.41**.

Serial serum and plasma samples were taken:- before surgery, at end of surgery, and on post-operative days 1, 2, 3 and 4. Liver tissue samples were taken at the beginning and end of surgery. Details are given in **Section 4.4.4.**

5.4.2 Efficacy Variables

The following biomarkers were measured in this study and details of laboratory methods are given below.

- Inflammation: IL-6, IL-10, TNFα
- Adipocytokines: Leptin, Resistin and Adiponectin
- Apoptosis: CK-18 fragments, TRAIL and Fas Ligand
- Oxidative stress: TBARS, SOD, GPX, protein carbonyls

These markers were measured using commercially available assays, according to the manufacturer's instructions. All samples assayed were run in duplicate and their concentrations were determined, with the aide of the manufacturer's standards, provided with each kit.

5.5 Detailed Laboratory methods

The laboratory methods are divided into three separate sections: Markers of Oxidative Stress, Cytokines and Markers of Liver injury. Please note that all materials and disposable laboratory equipment (for example, sample tubes) was sourced from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Poole, UK).

5.5.1 Markers of Oxidative Stress

Markers measured included a marker of Lipid Peroxidation TBARS, Superoxide Dismutase, Protein Carbonyls and Glutathione Peroxidase.

5.5.1.1 TBARS Assay

The Oxitek TBARS Assay Kit from Enzo Life Sciences was used in this study (Enzo Life Sciences UK, Ltd, Exeter, UK). The oxidised lipid components of the serum are first precipitated out of solution before being resuspended and mixed with TBA. TBA forms a compound with malondialdehyde (MDA), the marker of lipid peroxidation, in a 2:1 ratio. The protocol has been modified below to allow multiple assays to be performed simultaneously in a 96-well plate. Therefore the quantities of reagents and sample were scaled down. The manufacturer states that normal plasma TBARS is ~ 1.5 MDA unit and serum TBARS is ~ 2.0 MDA units (measured in nmol/ml). Published values include: plasma mean 2.95 ±SEM 0.32 nmol/ml in lean controls with BMI 22.4 ±0.5 kg/m^2 and mean 14.6 ±0.5 nmol/ml in patients undergoing RYGB with mean BMI 48 ±1.9 kg/m^2 **(113)**; serum 1.8±0.9 nmol/ml in controls without liver disease and 3.4 ±1.3 nmol/ml in patients with NASH with mean BMI 33 ±4 kg/m^2 **(144)**; plasma mean 3.94 ±0.42 nmol/ml in lean controls with BMI 22.2 ±0.4 kg/m^2 and mean 19.2 ±1.2 nmol/ml at baseline in patients undergoing RYGB with mean BMI 47.1±1.5 kg/m^2 **(387)**.
Sample timepoints used for TBARS assay were Pre-operative, end of surgery, day 1, day 3.

5.5.1.1.1 Reagents and Standards used in the assay

Thiobarbituric acid (TBA) vials

TBARS diluent 1 – contains acetic acid
TBARS diluent 2 – contains sodium hydroxide
Sodium dodecyl 142 sulphate (SDS) solution
MDA Diluent – sterile deionized water
Sodium Heparin Solution 40,000units/ml
Manganese Chloride Solution
PBS

MDA Standard – solution containing 100nmol/ml Malondialdehyde Bis (dimethyl acetal)

5.5.1.1.2 Reagents, Standards and Sample Preparation

TBA/Buffer Reagent: 100mls of TBARS Diluent 1, 100mls of TBARS Diluent 2 and 2 vials of TBA were mixed carefully together until all the TBA was dissolved. This allowed enough reagent for 80 reactions.

MDA Standards: A series of 5 standards were created by serial dilution in MDA Diluent. Undiluted MDA standard was used for the 100nmol/ml standard. Subsequent dilutions were created as follows: 50nmol/standard – 500 µl MDA in 500 µl diluent; 25nmol/ml standard – 250 µl MDA in 750 µl diluent; 12.5 nmol/ml standard – 125 µl MDA in 875 µl diluent. The 0 standard was 1000 µl diluent alone.

First the oxidised low density lipoprotein (LDL) and very low density lipoprotein (VLDL) must be precipitated from the serum. A sodium heparin solution containing 40,000units /ml and a 1.06M manganese chloride in water (5.25g in 25mls) were prepared. A working reagent solution of 300µl sodium heparin and 5mls manganese chloride was prepared. 0.5mls of sample serum was added to 0.5mls isotonic saline to produce a sample solution. 100µl working solution was added to 1ml sample solution and vortexed. The mixture was allowed to incubate at room temperature for 15minutes to allow precipitation of the LDL/VLDL. The mixture was then centrifuged for 15minutes at 8000rpm. The supernatant was decanted and the pellet was re-suspended in 0.5ml PBS.

5.5.1.1.3 Assay procedure

50 µl of sample solution (see 4.6.7) was added to each well, along with 50 µl SDS solution. The solutions were mixed by swirling. 1.25mls TBA/Buffer Reagent was added forcefully into each well. The plate was incubated at 95°C for 60minutes. At the end of this period, the plate was cooled to room temperature in an ice bath. The plates were centrifuged at 3000rpm for 15minutes. 300 µl supernatant was removed from each well and placed in a fresh 96-well plate for analysis.
Each sample was assayed in duplicate. Five standards from 0 to 100nmol/ml concentrations were also assayed in duplicate on each plate.
Absorbance was read using a spectrophotometer at 532nm (Dynex MRX, Dynex technologies Ltd. Worthing, UK).

A standard curve was created. Curve fitting settings included Lin/Lin axes scaling and Linear regression with data extrapolation was performed. Values of absorbance were interpolated from the generated standard curve.
An example of the standard curve is given below. The R-squared value was ≥0.996 for each plate.

5.5.1.2 Superoxide Dismutase ELISA

SOD measurement was performed using a commercially available kit: Cu/Zn SOD Platinum Enzyme Linked Immunosorbent Assay (ELISA) from Alexis Biochemicals (purchased from Enzo Life Sciences UK, Ltd, Exeter, UK). This is a quantitative sandwich enzyme immunoassay technique. An anti-human Cu/ZnSOD antibody coating is adsorbed onto microwells. The manufacturer states that the normal range is 0.226 mg/L (range not detectable – 0.352).

Sample timepoints used for SOD ELISA were Pre-operative, end of surgery, day 1, and day 3.

5.5.1.2.1 Reagents and Standards used in ELISA

Manufacturer's 96-well microwell plate with monoclonal antibody to human Cu/ZnSOD
HRP-Conjugate anti-human Cu/ZnSOD monoclonal antibody
Human Cu/ZnSOD Standard solution – 5ng/ml
Assay Buffer (PBS with 1%Tween20 and 10% BSA)
Phosphate Buffered Saline
Wash Buffer (PBS with 1% Tween20)
Substrate solution of tetramethyl-benzidine
Stop solution (Phosphoric acid 1M)
Deionized Water

5.5.1.2.2 Reagents, Standards and Sample Preparation

Wash buffer and Assay Buffer concentrates were diluted to working volume with deionized water to produce a final volume of 1000mls of Wash Buffer and 100mls of Assay Buffer.
HRP-Conjugate was diluted 1:5 by adding 20 µl to 80 µl of Assay Buffer. A further 1:100 dilution was made by adding 0.06mls of above to a further 5.94mls Assay Buffer.
Standards were prepared by serial 1:2 dilutions with addition of 100 µl PBS to 100 µl of each successive dilution of 5ng/ml Standard solution, leaving 7 standards:- 5ng/ml, 2.50ng/ml, 1.25ng/ml, 0.63ng/ml, 0.31ng/ml, 0.16ng/ml, 0.08ng/ml and a blank standard of PBS alone.

Sample aliquots were thawed and kept on ice until it was time to perform the assay. Subsequently they were allowed to come up to room temperature. Samples of serum were diluted 1:20 with PBS, using 10 µl of sample to 190 µl of PBS.

5.5.1.2.3 Assay Procedure

The microwell plate was washed twice with 400 µl of Wash Buffer per well, with thorough and careful aspiration of the microwell contents between washes. The Wash Buffer was allowed to sit in the wells for approximately 15 seconds on each occasion. Excess Wash Buffer was removed by tapping the well upside down on an absorbent pad.

100 µl PBS was added in duplicate to the blank wells. 90 µl PBS was added to all other wells. 10 µl of each sample and standard were loaded onto the 96 well pre-coated microwell plate in duplicate according to a predetermined template, for ease of sample identification.

50 µl of HRP-Conjugate was added to all wells. The plate was covered with an adhesive film and incubated on a microplate shaker set at 100rpm, for 1 hour at 25°C.

At the end of this period, the wells were emptied and washed 3 times with 400 µl Wash Buffer for each wash as before.

100 µl of Substrate solution was pipetted into each well. Again the plate was incubated for 10mins at room temperature, in a darkened part of the room.

Once the highest standard had become a dark blue colour, the reaction in all wells was stopped by adding quickly 100 µl of Stop Solution to each well. Great care was taken to perform this step quickly and consistently.

Absorbance of each well was read by a spectrophotometer using 450nm as the primary wave length (Dynex MRX, Dynex technologies Ltd. Worthing, UK).

A standard curve was constructed using a cubic spline technique and the concentration of SOD in each well was interpolated from the standard curve. An example of the standard curve is given below. The R^2 value was ≥0.995 for each plate.

5.5.1.3 Protein Carbonyl ELISA

This is another commercially available ELISA (purchased from Enzo Life Sciences UK, Ltd, Exeter, UK). In measuring PC, the assumption is made that there are no nucleic acids (and therefore no nuclear material) within the sample, as these would give falsely elevated readings. PC quantification can occur by various

methods, including Western blotting and HPLC. In this study, we employed the simplest technique, using a sandwich enzyme technique, based on the derivatisation of the Carbonyl side chains with dinitrophenylhydrazine (DNP), which can then be detected using anti-DNP antibodies. PC are measured in nmol/mg protein and published values include: 0.7 nmol/mg protein in lean controls with mean BMI 25 kg/m^2 and 1 nmol/mg protein in obese patients with mean BMI 48 kg/m^2 before bariatric surgery (values estimated visually from graph) **(371)** and 0.73 ±0.13 nmol/mg in controls with BMI 24.4 ±2.5 kg/m^2 and 1.13 ±0.21 nmol/mg in obese patient before AGB with mean BMI 48.4 ±6.4 kg/m^2 **(372)**.

Sample timepoints used for PC ELISA were Pre-operative, day 1 and day 3.

5.5.1.3.1 Reagents and Standards used in ELISA

SOD ELISA 96 microwell plate
EIA Buffer
Blocking reagent
Dinitrophenylhdrazine (DNP)
Guanidine hydrochloride diluent
Anti-DNP-biotin antibody
Streptavadin-horseradish peroxidase
Chromatin Reagent
Stopping Reagent
Standards – premixed at 6 concentrations
Carbonyl control sample

5.5.1.3.2 Reagents, Standards and Sample Preparation

2ml of EIA Buffer was added to the blocking reagent container, mixed and then diluted in a larger container to a final volume of 75ml.

0.5ml of this diluted blocking solution was added to the Anti-DNP-biotic antibody microvial and mixed well, then made up to a total of 20ml with more diluted blocking solution.
0.5ml of diluted blocking solution was added to the Streptavidin-HRP microvial, mixed well and then made up with more solution to a total of 20ml.
1ml of DNP was added to 9ml guanidine hydrochloride.
The oxidised protein standards (1-6) were each mixed with 25 µl deionised water, vortexed and then kept overnight (for 13 hours total) at 37 °C. Before use on the morning of the experiment, they were revortexed to ensure the whole sample was dissolved.

5 µl of each sample was added to a 1.5ml reaction tube containing 200 µl of diluted DNP solution, mixed well and incubated for 45minutes at room temperature. 5 µl of each sample/diluted DNP mixture was then added to another reaction tube, containing 1ml of EIA buffer. 200 µl of each sample was loaded into the 96 well ELISA microplate in duplicate, according to a pre-assigned plate map. The plate was left overnight (for 13 hours in total) at 4 °C.

5.5.1.3.3 Assay Procedure

The plate was allowed to come to room temperature and washed, five times with a volume of 300 µl per well of EIA buffer. After emptying the wells by tapping on a piece of blotting paper, 250 µl of diluted blocking solution was added to each well. The plate was incubated for 30 minutes at room temperature. The plate was washed again, as above. 200 µl of diluted anti-DNP-biotin antibody was added to each well and the plate was incubated for 1 hour at 37 °C. The plate was washed again, as above. 200µl diluted streptavidin-HRP was added to each well and incubated for 1 hour at room temperature. Further washings occurred, as above.
200 µl Chromatin reagent was added to each well and the colour development in the wells was observed. The plate was kept at room temperature and absorbance at 650nm was measured repeatedly with the spectrophotometer every 1-2minutes until, at approximately 13 minutes after adding Chromatin, the highest standard had an absorbance of approximately 0.7. At this point the reaction was arrested by

adding 100 µl of Stopping Reagent to each well. Absorbances were read at 450nm (Dynex MRX, Dynex technologies Ltd. Worthing, UK).
A standard curve was constructed by the software, using the Lin/Lin setting, with linear regression. R^2 was 0.92 for plate 1 and 0.96 for plate 2. PC concentrations were interpolated from the standard curve.

5.5.1.4 Glutathione Peroxidase Activity

This assay works by creating conditions whereby the activity of GPX within a sample is measuring by a change in the absorbance of the admixture at 340nm. It is a dynamic assay. The kit used was the AssayDesigns GPX Activity Kit (purchased from Enzo Life Sciences UK, Ltd, Exeter, UK). The manufacturer states the activity is measured as units/ml. They do not provide a standard reference range.

Sample timepoints used were pre-operative, end of surgery and day 1.

5.5.1.4.1 Reagents used in ELISA

Assay Buffer
GSH + NADPH
Glutathione Reductase
Glutathione Peroxidase
Cumene Hydroperoxide
Distilled water
Peroxide-free Triton X-100

5.5.1.4.2 Preparation of Reagents and Samples

In order to run 2 plates (160 samples), a Reaction Mixture was created by mixing 440 µl Glutathione Reductase with 440 µl Reconstituted GSH + NADPH and adding this to 3.520ml Assay Buffer. The mixture was stored on ice until use.
Cumene Hydroperoxide was aliquoted and kept on ice. It was brought to room temperature just before use.

An aliquot of each sample was thawed and kept on ice until use.

5.5.1.4.3 Assay Procedure

GPX activity is a function of the fall in absorbance of the reaction mixture over a period of 15 minutes (discussed further below). Therefore the plate reader was set up to perform absorbance measurements every minute for a period of 15minutes (Dynex MRX, Dynex technologies Ltd. Worthing, UK). The microplate was set-up by adding 140 µl Assay Buffer, 20 µl of the Reaction Mixture and 20 µl of sample. Assays were performed in duplicate. The reactions were initiated by adding 20 µl Cumene Hydroperoxide to each well quickly using a multichannel pipettor. Absorbance was measured immediately at 1 minute intervals for 15 minutes. A set of controls were loaded where a further 20 µl Assay Buffer was added in place of the sample volume. A background rate of decay in absorbance was provided by the controls.

Glutathione Peroxidase activity was determined by using the optical plate reader software. The gradient of the decay slope of absorbance at 340nm was determined. The slope of the controls was subtracted from this to give a net change in absorbance per minute (ΔA340/min). One unit of GPX is defined as the amount of enzyme that will cause the oxidation of 1 nmole of NADPH to NADP per minute at room temperature. The reaction rate could then be determined using an adjusted extinction coefficient of NADPH, 0.00379µM^{-1}.

GPX activity in Units/ml $= \frac{nx\Delta A340/\min}{0.00379\mu M-1} X \frac{0.2ml}{180\mu l}$

where volume of sample in each well was 180µl, using undiluted samples.

5.5.2 Cytokines

This section is divided into Luminex Multiplex Cytokine Array, which was used to measure TNFα, IL-6, IL-10 and the adipocytokines Leptin, Resistin and Adiponectin, and TNFα High Sensitivity ELISA. TNFα was measured in two ways due to problems with its detection within the study samples.

5.5.2.1 Luminex Multiplex Cytokine Array

The kit used was Fluorokine® MultiAnalyte Profiling Kit (R&D Systems Europe Ltd, Abingdon, UK) and the results were read on a Luminex® Analyzer (Luminex B.V., Oosterhout, The Netherlands). This is a commercially available bead-based assay, dependent on a flow-based sorting and detection platform akin to flow cytometry. Microparticles pre-coated in various analyte-specific antibodies are added into samples and incubated to allow binding of target analytes to its corresponding microparticle. A biotinylated antibody cocktail is then added, which also binds to the microparticle-bound analyte. Finally a streptavidin-bound chromogen (in this case, phycoerythrin) is added to bind onto the captured biotinylated antibody. The Luminex analyser is able to scan the microparticles in each well with two lasers: one detects the identity of the analyte and the other measures the magnitude of the colour signal derived from the phycoerythrin, which is proportional to the concentration of the bound analyte. The main advantage is that multiple different analytes can be measured from one sample by simply adding the corresponding antibody-coated microparticles at the beginning of the assay. In this case, a commercially available Obesity Panel had already been tested to exclude interference and cross-reactivity between the analytes of interest.

Sample timepoints used for the Luminex multiplex assay were Pre-operative, end of surgery, days 1-3.

5.5.2.1.1 Reagents used in the assay

Calibrator Diluent RD6-46 - buffered protein base with preservatives
Wash Buffer – buffered surfactant with preservatives
Biotin Antibody Diluent 2 - buffered protein base with preservatives
Streptavidin-Phycoerythrin (PE)
Standard Cocktail of recombinant human protein biomarkers in a buffered protein base with preservatives
Microparticles for each analyte
Microparticle Diluent 4 – buffered protein base with preservatives
Deionised water

Luminex 96-well filter-bottomed microplate

5.5.2.1.2 Standards used in the assay

Standard Cocktail was reconstituted with Calibrator Diluent according to the individual instructions in each assay – approximately 1ml volume total.
Using 7 polypropylene mini-tubes for the serial dilution of the Standard, 500 µl of reconstituted Standard was pipetted into Standard 1 tube. 200 µl of Calibrator Diluent was put in the remaining six tubes. A 3-fold dilution series was produced by transferring 100 µl from Standard 1 into Standard 2, mixing thoroughly then transferring 100 µl from Standard 2 to Standard 3 and so on. Each Standard Cocktail was different and the values of the highest Standard were recorded on a card for each plate.

5.5.2.1.3 Reagent Preparation

Wash Buffer was reconstituted with deionised water 1:25 to create 500ml of Wash Buffer.
Each Microparticle Concentrate vial was centrifuged for 30 seconds to clear any particles from inside the cap and then vortexed to resuspend the particles. 50 µl of Microparticle Concentrate was added to 5.0 ml Microparticle Diluent. The diluted Microparticles were shielded from light until use.
As above, the Biotin Antibody Concentrate vials were centrifuged and vortexed before diluting 50 µl in 5.25ml Biotic Antibody Diluent.
Streptavidin-PE vial was also centrifuged then vortexed. A 100-fold dilution was performed by adding 55 µl Streptavidin-PE to 5.5ml Wash Buffer. The diluted solution was shielded from light with aluminium foil.

5.5.2.1.4 Sample Preparation

The initial plan had been to assay all six of the target cytokines on one plate, as marketed by the manufacturer. The printed protocol advised a four-fold dilution of each sample with Calibrator Diluent. In fact, the relative abundances of TNFα, IL-6, IL-10, Leptin, Resistin and Adiponectin differed greatly, and it was noted that

the Standard Cocktail provided with each plate meant that individually optimised Standard curves could not be produced for each analyte. Therefore three microplates were required for each set of samples. The combinations and dilutions are given as follows: TNFα, IL-6 and IL-10 – a 1:1.5 dilution, using 100 µl sample and 50 µl Calibrator Diluent; Leptin and Resistin – a 1:5 dilution, using 30 µl sample and 120 µl Calibrator Diluent; Adiponectin – a 1:100 dilution, using 10 µl sample and 190 µl diluent. Diluted Samples were stored on ice until ready for use.

5.5.2.1.5 Assay procedure

The filter-bottomed 96-well microplate was pre-wet with 100 µl of Wash Buffer and the liquid removed using the Vacuum Manifold. After resuspending the Microparticle mixture by vortexing, 50 µl of each individual analytes Microparticle Mixture was added to each well.

50 µl of Standards and Diluted Samples were added in duplicate to the wells according to a predetermined plate map. The microplate was securely covered with a foil plate sealer and incubated for 3 hours at room temperature on a horizontal orbital plate shaker (0.12" orbit) set at 500 rpm.

At the end of this period, the microplate was emptied using the Vacuum Manifold then each well was washed with 190 µl of Wash Buffer. The liquid was emptied again using the Vacuum Manifold. This washing procedure was performed 3 times in total. At the end, complete removal of the liquid was checked in each well.

50 µl of diluted Biotic Antibody Cocktail was added to each well. After covering with a foil plate sealer, the microplate was incubated for 1 hour at room temperature on the orbital shaker.

Three washes were performed as detailed above.

50 µl of diluted Streptavidin-PE was added to each well. The microplate was covered and incubated on the orbital shaker as above for 30 minutes.

Three washed were performed as above.

The microparticles were resuspended by adding 125 µl Wash Buffer to each well (an increased volume to avoid damaging the needle on the Luminex Bioanalyser).

The microplates were read on the Bioanalyser.

Standard curves were generated using the analyser computer software. A 5-PL curve fit was used to produce the curve and the concentrations of analytes in each sample were interpolated. The final results were corrected for the dilution factor.

5.5.2.2 Tumour Necrosis Factor α High Sensitivity ELISA

Despite adjusting the dilution factor, the range of serum TNFα concentrations in this patient population was still centred on the value of the lowest standard in the Luminex Assay above, and it was felt that an additional assay should be used to confirm the validity of the results. Therefore, a high-sensitivity ELISA was used, Human TNFα High Sensitivity Quantikine Assay (R & D Systems Europe Ltd, Abingdon, UK). This is a quantitative sandwich enzyme immunoassay employing a recombinant E.coli-derived human anti-TNFα antibody. The assay detects Total circulating TNFα, including both free TNFα and TNFα bound to soluble receptors. Sample timepoints used for the TNFα ELISA were Pre-operative, end of surgery, day 1 and day 3.

5.5.2.2.1 Reagents used in the assay

Anti-TNFα antibody coated 96-well Microplate
TNFα Conjugate Antibody – conjugated to alkaline phosphatase
TNFα Standard
Assay Diluent RD1F – buffered protein base with preservatives
Calibrator Diluent RD6-13 - buffered protein base with preservatives
Wash Buffer
NADPH Substrate – lyophilised NADPH with stabilisers
Substrate Diluent - buffered solution with stabilisers
Amplifier – lyophilised amplifier enzymes with stabilisers
Amplifier Diluent – buffered solution containing INT-violet with stabilisers
Stop Solution – sulphuric acid 2N
Deionised water

5.5.2.2.2 Reagent, Standards and Sample Preparation

Wash buffer was reconstituted with deionised water to create 1000ml solution.
Substrated Solution was made by reconstituting the vial of lyophilised Substrate in 6ml Substrate Diluent. Similarly lyophilised Amplifier was diluted in 6ml Amplifier Diluent.

TNFα Standard was reconstituted with Calibrator Diluent, making a 32pg/ml stock solution. After allowing the solution to sit for 15minutes, a serial 1:2 dilution was performed. 500 µl Calibrator Diluent was pipetted into 6 marked polypropylene tubes. 500 µl TNFα Standard was transferred into the first tube. The solution was gently mixed and a further 500 µl was transferred to the next tube, and so on. Eight standards were created: 32pg/ml, 16pg/ml, 8pg/ml, 4pg/ml, 2pg/ml, 1pg/ml, 0.5pg/ml and plain Calibrator Diluent served as the zero standard.

Sample serum aliquots were thawed on ice and brought to room temperature just before use. They were added to the reaction mixture undiluted.

5.5.2.2.3 Assay procedure

The whole procedure was performed with facemask and gloves to avoid contamination with saliva and respiratory secretions, which are rich in TNFα.
50 µl Assay Diluent was added to each well, after vortexing diluent to ensure mixing. 200 µl of Sample and Standards were added in duplicate according to a predetermined plate map. The microplate was covered with adhesive film incubated for 3 hours at room temperature.
After this period, the wells were aspirated and excess liquid was removed by tapping the inverted plate vigorously on blotting paper. Each well was filled with 400 µl Wash Buffer using an automated manifold dispenser. The wells were again emptied as before. 5 further wash steps were performed as above. After the sixth occasion, any excess liquid was removed by vigorous inversion onto blotting paper as above.
200 µl TNFα HS Conjugate was added to each well. The plate was incubated for 2 hours at room temperature on the benchtop.
6 Washes were performed as above.

50 µl Substrate Solution was added to each well and the plate was incubated for 1 hour as above.

50 µl Amplifier Solution was added to each well and a further 30 minute incubation took place.

At the end of this period, 50 µl Stop Solution was added to each well.

Optical density of each well was measured using a microplate reader at 490nm, with λ correction at 650nm (Dynex MRX, Dynex technologies Ltd. Worthing, UK). A standard curve was generated, using logarithmic transformation and regression, using a Spline Smoothed fitting algorithm. TNFα concentrations were interpolated from the standard curve.

Unfortunately, >50% of the wells did not give a reading within the range of the standard curve and therefore these results were not used. It was not possible to repeat the ELISA due to lack of samples.

5.5.3 Markers of Liver Injury

This section is divided into sections for Cytokeratin-18, TRAIL and Fas-Ligand, and Apoptosis Proteome Profile Tissue Array.

5.5.3.1 CytoKeratin-18 ELISA

CK-18 M65 and M30 Assays are solid-phase sandwich enzyme immunoassays, using a horseradish peroxidase enzymatic reaction to give rise to a change in colour and absorbance of the reaction mixture, which is proportional to the concentration of M65 or M30 in the sample (M65® Classic and M30 Apoptosense®, PEVIVA AB, Bromma, Sweden, supplied through BIOAXXESS® UK, Malvern, UK). Protocols for these assays are very similar and therefore recorded together below, to avoid duplication. Differences between assays are indicated.

Sample timepoints used for CK-18 M65 and M30 assay were Pre-operative, day 1, day 2 and day 4.

5.5.3.1.1 Reagents and standards used in the assays

M65 and M30 Coated 96-well Microplates
M65 and M30 HRP Conjugate –vial
Conjugate Dilution Buffer - phosphate buffer with stabilisers
M65 and M30 Standards (7 each assay)
Tetramethylbenzidine (TMB) Substrate
Stop Solution – sulphuric acid
Wash Buffer solution

M65 Standards – 7 provided with values (Units/L): 0, 125, 240, 500, 750, 1200
M30 Standards – 7 provided with values (Units/L): 0, 75, 150, 500, 750, 1000

5.5.3.1.2 Reagent and Sample Preparation

M65 and M30 HRP Conjugates were diluted by adding 0.4ml of HRP Conjugate to 9.2ml of Conjugate Dilution Buffer.
All reagents were brought to room temperature just before use.
Sample aliquots were thawed on ice and brought to room temperature just before use.

5.5.3.1.3 Assay procedure

25 µl of samples, standards and controls were added to the 96-well microplates in duplicate according to a predetermined plate map.
75 µl of diluted HRP Conjugate solution was added to each well.
M65 microplate was incubated for 2 hours on a shaker at 600rpm at room temperature; M30 microplate was incubated for 4 hours on a shaker at 600rpm.
At the end of the incubation period, the microplate was aspirated and then washed 5 times with 250ml of Wash Buffer per well each time.
200 µl of TMB was added to each well and the microplate was incubated in darkness for 20 minutes.
50 µl Stop Solution was added to each well.

After mixing for 10 seconds, the absorbance for each plate was read after 5 minutes at 450nm on a Microplate reader (Dynex MRX, Dynex technologies Ltd. Worthing, UK).
A Standard Curve was generated, using Lin/Lin axes scaling and a Cubic Spline algorithm. Concentrations of Ck-18 M65 and M30 were determined by interpolating the values from the Standard Curve. R^2 was 1.000.

5.5.3.2 TRAIL and FasLigand ELISA

Both ELISAs are sandwich enzyme immunoassay based on a horseradish peroxidase reaction, which leads to a change in absorbance of the reaction mixture proportional to the concentration of TRAIL or FasL in the sample. The assay protocols are very similar and therefore are included together below. The kits used were Quantikine ELISA (R & D Systems Europe Ltd, Abingdon, UK). The manufacturer states that normal range of TRAIL in serum is mean 76 pg/ml (range 28-135) and of Fas Ligand is mean 85.2 ±SEM 25.6 pg/ml (range 39.8 – 145).

Sample timepoints used for TRAIL assay were Pre-operative, end of surgery, day 1, day 2 and day 4.

5.5.3.2.1 Reagents used in the assay

FasL and TRAIL 96-well microplates (coated with mouse monoclonal antibody to FasL or TRAIL)
Fas L and TRAIL HRP-Conjugate
Standards
Assay Diluent RD1S – buffer with preservatives
Calibrator Diluent RD5K – buffered protein base with preservatives
Tetramethylbenzidine Solution in two parts - stabilised TMB and stabilized hydrogen peroxide
Cell Lysis Buffer – buffered solution with preservatives
Wash Buffer – buffered surfactant with preservatives

Stop Solution – sulphuric acid 2N
Deionised water

5.5.3.2.2 Reagents, Standards and Sample preparation

All reagents were brought to room temperature before use. Wash and Cell Lysis buffer concentrates were diluted with deionised water. All procedures were performed whilst wearing a facemask to avoid contamination as high levels of TRAIL are found in saliva and respiratory secretions. The TMB-Substrate solution was reconstituted by adding 12.5ml of TMB to 12.5ml hydrogen peroxide for each assay.

Both standards were reconstituted with 1ml deionised water. This produces a stock solution of 10,000pg/ml. The standards were allowed to sit for 15minutes before proceeding.
For FasL, seven test tubes for serial dilution were used. The first was filled with 360 µl Calibrator Diluent ED5K, with 200 µl Calibrator Diluent in the remaining 6 tubes. 40 µl of the stock Standard solution was put in the first tube, forming the 1000 pg/ml standard (highest). After mixing, 200 µl was taken from this tube and put in the next one. The procedure was repeated for the next 5 tubes forming standards 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.5 pg/ml and 15.6 pg/ml. Plain Calibrator Diluent served as the zero standard.
TRAIL standards were created in the same manner, except the first tube was filled with 900 µl Calibrator Diluent, with 1000 µl in each subsequent tube. Then 100 µl of TRAIL standard stock solution was transferred to the first tube with 500 µl transferred to the next 6 tubes serially. The concentrations of TRAIL standard were 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.5 pg/ml and 15.6 pg/ml. Plain Calibrator Diluent served as the zero standard.

Serum sample aliquots were thawed on ice. For TRAIL assay, samples were diluted 2-fold with 100 µl sample diluted in 100 µl Calibrator diluent in a separate 96-well plate. They were then transferred to the ELISA plates in duplicate, according to a predetermined plate map.

5.5.3.2.3 Assay procedure

100 µl Assay Diluent RD1S was added to each well. 50 µl of sample and standard was added to each well, in duplicate according to a plate map. (The TRAIL sample had been diluted two-fold). The microplates were incubated for 2 hours at room temperature, with the TRAIL plate placed on a horizontal shaker set at 500 rpm.
At the end of this period, the wells were aspirated and washed four times, with a volume of 400 µl Wash Buffer per well per wash. After the last wash, the plates were inverted forcefully onto blotting paper to ensure all Wash Buffer had been removed. 200 µl of the relevant HRP-Conjugate was added to each well. The plates were again incubated for 2 hours at room temperature, with the TRAIL plate placed on a shaker as before. The washing step was repeated as above. 200 µl of the relevant TMB-Substrate solution was added to each well. Both plates were mixed gently and incubated for 30 minutes at room temperature on the benchtop.
At the end of this period, 50 µl Stop Solution was added to each well and the plates were gently shaken to ensure mixing.
The absorbance was read by a microplate reader at 450 nm with a λ correction at 550 nm (Dynex MRX, Dynex technologies Ltd. Worthing, UK). The standard curves were generated using linear regression with Log/Log axes scaling. R^2 was 0.99 for both plates. Concentrations of FasL and TRAIL were interpolated from the standard curves.

5.5.3.3 Apoptosis Proteome Profile Tissue Array

After tissue damage, the expression of various apoptosis-associated proteins changes. Analysing these expression profiles may give an indication of the mechanisms involved in causing the tissue damage and also possible target proteins affected by the administration of N-Acetylcysteine. This Apoptosis Proteome Profile Array (Apoptosis Array, AA) allows rapid, simultaneous detection of 35 apoptosis-related proteins in one assay (Proteome Profiler Human Apoptosis Array Kit, R & D Systems Europe Ltd, Abingdon, UK). Relative quantification of each protein gives an indication of the intracellular processes occurring. The AA contains 35 spots coated with specific antibodies, which can bind the target proteins, along with controls. The original protocol was for use with cell lysates. Therefore an initial modification was performed to allow us to

use liver biopsy material. After incubating with cellular extracts from sample tissue, unbound proteins are washed off. Biotinylated detection antibodies are then applied to the AA, followed by a horseradish peroxidase-Streptavidin reagent, leading to the production of a chemoluminescent signal. The strength of this signal is proportional to the concentration of target protein and is captured photographically, before signal strength is measured by image processing software.

Methods of patient selection are given in Section **2.3.1**. There were enough kits for 14 patients' samples before and after surgery, 7 patients were selected randomly from each group. This was performed using an online random number generator (http://www.randomizer.org/form.htm).

5.5.3.3.1 Reagents used in the assay

HepG2 cells
EMEM medium
Phosphate buffered saline (PBS)
Aprotinin
Leupeptin
Pepstatin
Triton X-100
Deionised water
Human Apoptosis Array membranes
Array Buffers 1, 2 and 3
Lysis Buffer-15
Wash Buffer
Human Apoptosis Detection Antibody Cocktail
Streptavidin-Horseradish peroxidase solution
Photographic developing solutions

5.5.3.3.2 Standards used in the assay

Negative and Positive Control Spots were present on the Array membranes.

5.5.3.3.3 Reagent Preparation

Wash buffer concentrate was diluted by adding 40mls of buffer concentrate to 960ml deionised water. Array Buffer 1 was ready for use. 2 ml of Array Buffer 2 was added to 8ml of Array Buffer 3.
Leupeptin 25 µl, Pepstatin 5 µl and Aprotonin 50 µl were added to 5ml Lysis Buffer-15 to create sufficient Lysis Buffer for tissue homogenisation.

5.5.3.3.4 Sample Preparation

The protocol demanded that approximately 1 x 10^7 cells would be solubilised by 1ml Lysis Buffer. In order to determine how much liver tissue would be required for each assay, the weight HepG2 cells was used as a guide. HepG2 cells were resuspended in 1ml of EMEM medium. The cell count was 1.5 x 107/ml. After pelleting this cell solution, the estimated weight of 1 x 107 liver cells was 24mg. To solubilise these cells, approximately 42 µl Lysis Buffer was required for each milligram liver tissue (1000 µl lysis buffer ÷ 24mg tissue).
Each piece of liver biopsy tissue was weighed and rinsed with PBS. 42 µl/mg of Lysis Buffer was added along with Triton X-100 (1% of the total volume of Lysis Buffer ≈ 1.5 - 2.0µl). The cells were solubilised, then centrifuged at 10,000rpm for 5 mins at room temperature. The supernatant was decanted and put in a second microtube. A protein assay was performed modified Lowry method **(388)**.
Initially 5 µl of sample was diluted with 45 µl of PBS (1:10) but a further x3 dilution was performed, as initial protein levels were recordable. The original protocol indicated that each lysate should contain between 200 – 500 µg total protein in a volume of 250 µl. After determining the protein content of each sample lysate, the volume of lysate containing 350µg protein was calculated and the volume was made up to 250 µl with Lysis Buffer. Each sample was clearly marked and kept on ice until required.

5.5.3.3.5 Assay procedure

2ml of Array Buffer 1 was placed into each well and the array membrane was placed in the well facing upward. The well was incubated on a rocking shaker for 1 hour, with the well arranged so that the rocking occurred end-to-end. The sample

lysate was placed in a separate tube, with 1.25ml Array Buffer 1. The total quantity of diluted lysate was 1.5ml. The well was emptied of Array Buffer 1 and the diluted lysate was added to the well containing the array membrane. The well was covered and incubated overnight at 4 °C on a rocking platform shaker.

After a period of approximately 18 hours, the array was removed from the well and placed within an individual plastic tube container containing 20ml of Wash Buffer. To wash the array, the tube was placed on a rocking platform shaker for 10 minutes, before the Wash Buffer was exchanged for fresh Wash Buffer. Three such washes were performed. During this time, the well was washed thoroughly with deionised water and allowed to dry completely.

After 3 washes, the array membrane was removed from its washing container, shaken dry gently and placed back in the well. 15 µl of reconstituted Detection Antibody Cocktail was diluted by adding it to 1.5ml of Array Buffer 2/3. 1.5ml of this diluted Detection Antibody Cocktail was added to the well and incubated for an hour at room temperature on a rocking platform shaker.

At the end of this period, the array was removed from the well and washed again as above three times.

Again after shaking the array dry, it was placed back in the well. 1 µl of Streptavidin-HRP was diluted in 2 ml Array Buffer 2/3 and 1.5ml of this solution was added to the well with the array membrane. The array was incubated for 30 minutes on a rocking platform shaker at room temperature.

As above, the array membrane was removed and washed three times.

Excess Wash Buffer was drained off the membrane. The array membrane was placed on a plastic sheet protector and exposed to chemoluminescent reagents – Lumigen, according to the manufacturers protocol and transferred to a dark room.

In the dark room, the wrapped-up array membrane was placed on a photographic plate and exposed to X-ray film. After initial test runs, it was noted that 3minutes exposure time gave the best results.

The developed film was then scanned digitally. Using ImageJ software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland; http://imagej.nih.gov/ij/), the pixel density of each spot was measured, with the background value subtracted. The background value was determined by measuring the pixel density within the

negative control spots. Each protein was measured in duplicate so an average pixel density was calculated.

Each pair of liver biopsies was developed on the same film so that processing conditions were comparable. The fold change in pixel densities could be determined.

5.5.5 Statistical Analysis

Statistical analyses were performed using SPSS 20 (SPSS, USA).

The assumption of normality was tested for each parameter at baseline using the Shapiro-Wilk's test ($p>0.05$). All parameters were normally distributed at baseline. Outliers were also found in many of the parameters, as assessed by inspection of a boxplot. These outliers were left in the comparison. For SOD, two-way ANOVA using time and treatment group as fixed factors was not possible as the variances were unequal for. Thus, independent samples t-tests were used to compare values between groups at the various timepoints, with significance set at $p<0.05$. A secondary analysis of changes in parameters over time within groups was performed using repeated measures ANOVA. Post-hoc comparisons between different timepoints were performed after applying a Bonferroni correction.

For TBARS, GPX, PC and Adipocytokines, it was possible to perform Two-way ANOVA, using timepoint and treatment group as fixed factors.

IL-6, IL-10 and CK-18 fragment results were found to have unequal variances, so therefore two sets of analyses were performed as above.

TRAIL and FasL, the assumptions for Two-way ANOVA were satisfied and this test was used, using timepoint and treatment group as fixed factors.

Correlations were performed to assess the interaction between demographic and intraoperative factors and the primary outcome measures of the whole cohort grouped together. Where variables were non-normally distributed, they were log-10 transformed. Linearity between co-factors was assessed visually using scatterplots and Pearson's correlation was used.

Clinical significance was determined if $P<0.05$.

5.6 Results

There were no significant differences between treatment groups at baseline. Table 5.1 shows the values in each group. Biomarker levels were comparable to the published literature. Values for each timepoint are presented in Appendix B1.6 (Tables B.5 and B.6).

Table 5.1 Mean baseline values of biomarkers in treatment groups

Parameter	Control (n=10)	NAC (n=9)
	Mean ± SEM	
Oxidative Stress		
SOD (mg/L)	0.44 ±0.04	0.48 ±0.06
TBARS (nmol/ml)	19.4 ±3.9	13.4 ±4.3
GPX (U/ml)	7.37 ±1.7	9.77 ±1.7
PC (nmol/mg prot)	0.72 ±0.4	0.67 ± 0.3
Cytokines		
IL-6 (pg/ml)	5.22 ±0.7	7.49 ±1.5
IL-10 (pg/ml)	0.98 ±0.4	0.74 ±0.1
TNFα (pg/ml)	5.28 ±0.7	7.77 ±0.7
Leptin (ng/ml)	159 ±32	196 ±46
Resistin (ng/ml)	11.0 ±2.0	10 ±1.3
Adiponectin (ng/ml)	12231 ±2173	10007 ±2024
Apoptosis		
CK-18 M65 (IU/L)	315 ±60	378 ±82
CK-18 M30 (IU/L)	223 ±49	187 ±22
TRAIL (pg/ml)	101 ±9	90 ±11
Fas Ligand (pg/ml)	91 ±11	93 ±8

No significant differences between treatment groups at baseline ($p>0.05$)

5.6.1 Markers of Oxidative Stress

There were no significant differences between treatment groups in oxidative stress markers. SOD was the only parameter to change significantly from baseline ($p=0.001$), peaking by the end of surgery.

The wide variance in these data, with large confidence intervals and standard deviations indicate the difficulties in accurately assessing oxidative stress changes

in plasma and serum. A number of systematic biases may exist as slight variations in technique of sample collection and processing, such as minor differences in time from collection to freezing, may have had a large impact on the assay results. In the short term, no significant differences in the markers of lipid peroxidation (TBARS) and protein damage occur. Superoxide dismutase levels do rise following surgery but are not affected by NAC significantly (see Figure 5.5).

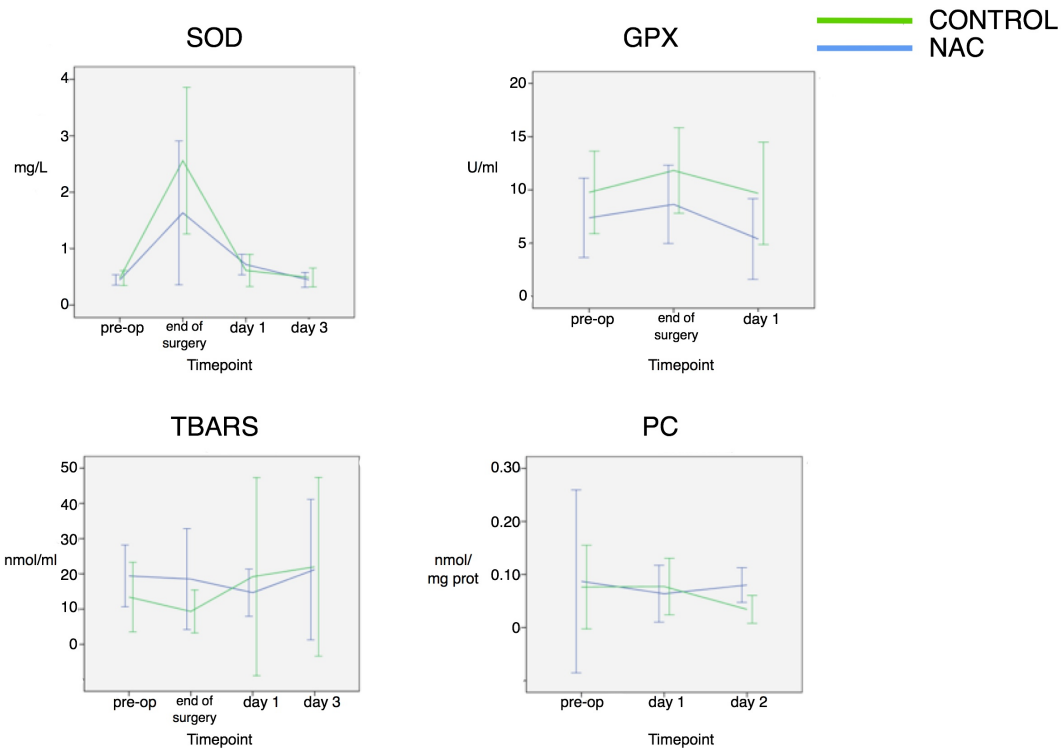


Figure 5.5 Graphs showing mean values (95% Confidence intervals) of markers of oxidative stress.
No significant differences between treatment groups (p>0.05). SOD levels rise by end of surgery significantly (p=0.001).

5.6.2 Cytokines

As shown in Figure 5.6, there are large changes in cytokines following surgery, with peak changes occurring within an hour of surgery in IL-6 and IL-10. TNFα levels are not significantly changed after surgery until day 2, when they fall to <50% of baseline (p=0.001). Changes in adipocytokines are more difficult to interpret and there are no significant differences within 24hours of surgery or between treatment groups (see Figure 5.7).

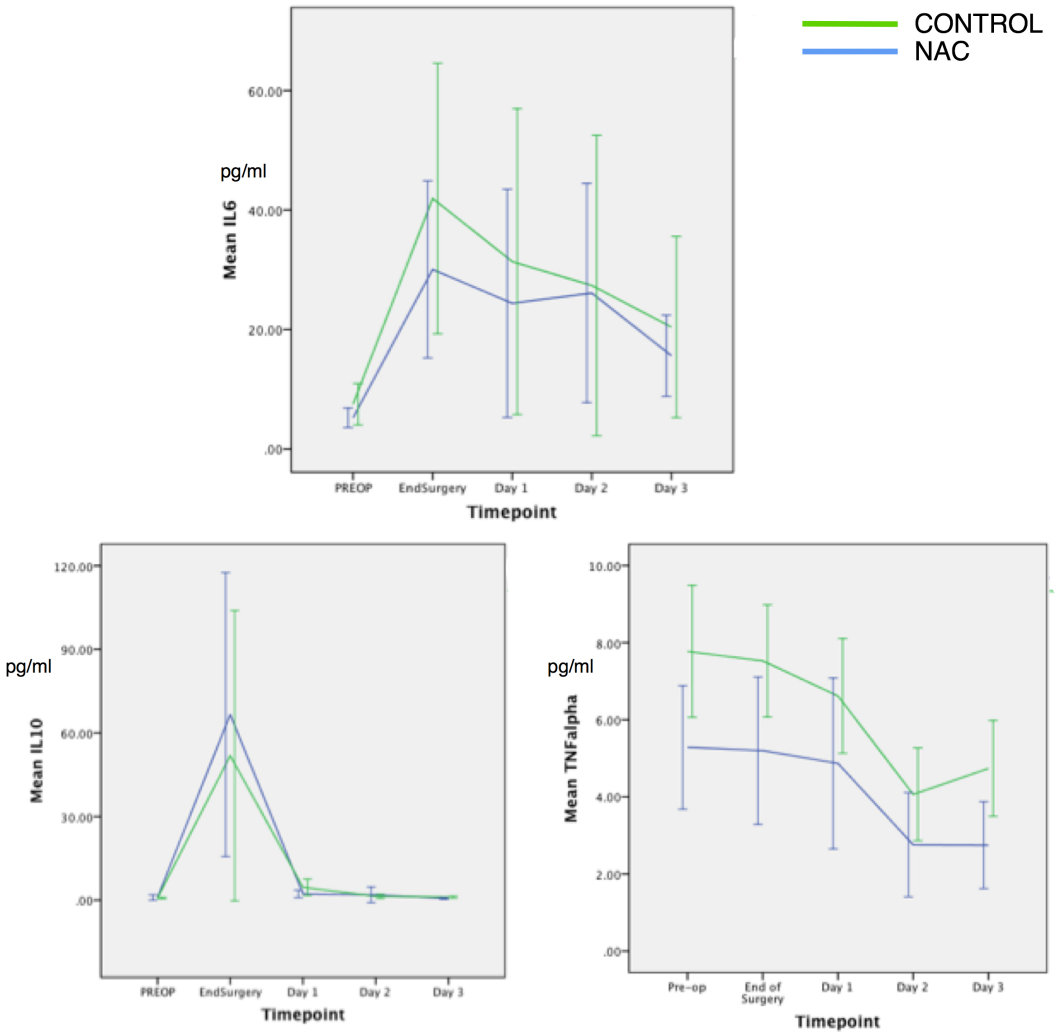


Figure 5.6 Graphs of Cytokines IL-6, IL-10 and TNFα following surgery.
No significant differences between treatment groups. Values shown are mean (95%CI) pg/ml.

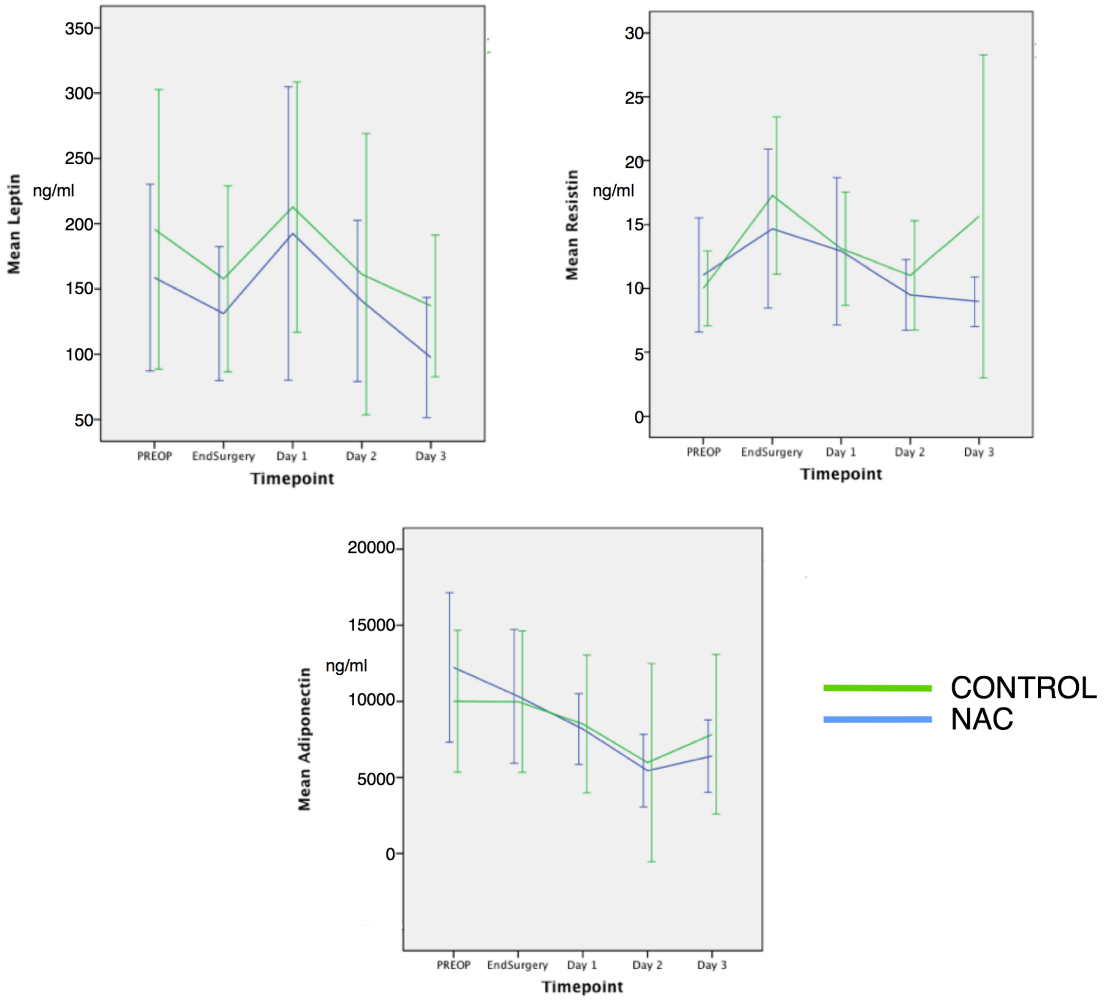


Figure 5.7 Adipocytokine changes after bariatric surgery.

Values of leptin, resistin and adiponectin are given as mean (95%CI) ng/ml. Note the different order of magnitudes used in the y-axes as adiponectin is >1000x more abundant than resistin. No significant differences between treatment groups ($p>0.05$).

5.6.3 Markers of Apoptosis

5.6.3.1 Cytokeratin-18 fragments, TRAIL, Fas ligand

There is a large rise in CK-18 post-operatively, indicating significant cellular damage (see Figure 5.8). Although there are no significant differences between treatment groups, these data do demonstrate that a significant degree of cell death occurs following surgery. Both necrosis and apoptosis occurs. TRAIL and FasL are not affected by NAC infusion and these data suggest they may not play a significant role in the cellular damage that occurs in this scenario (see Figure 5.8).

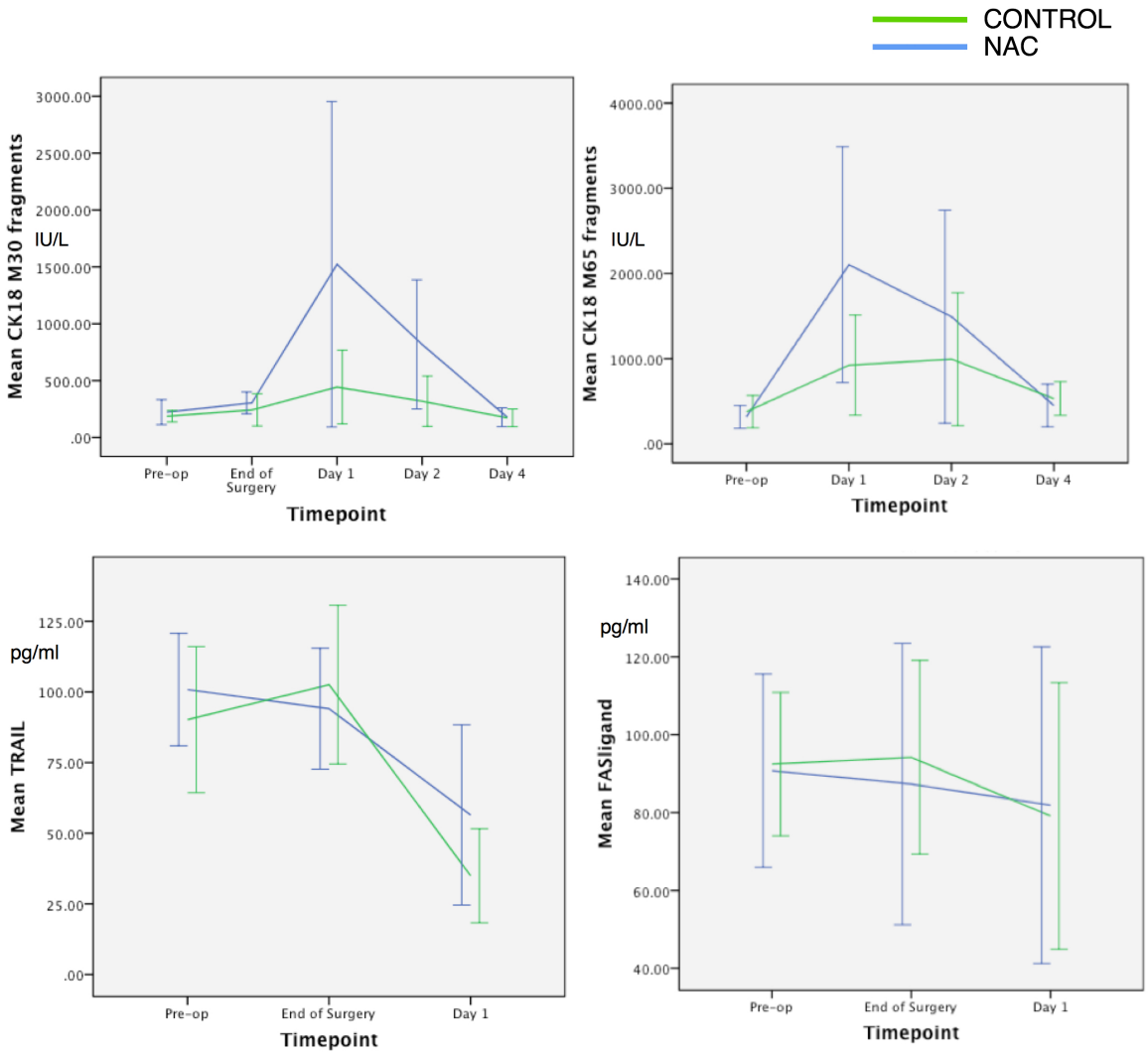


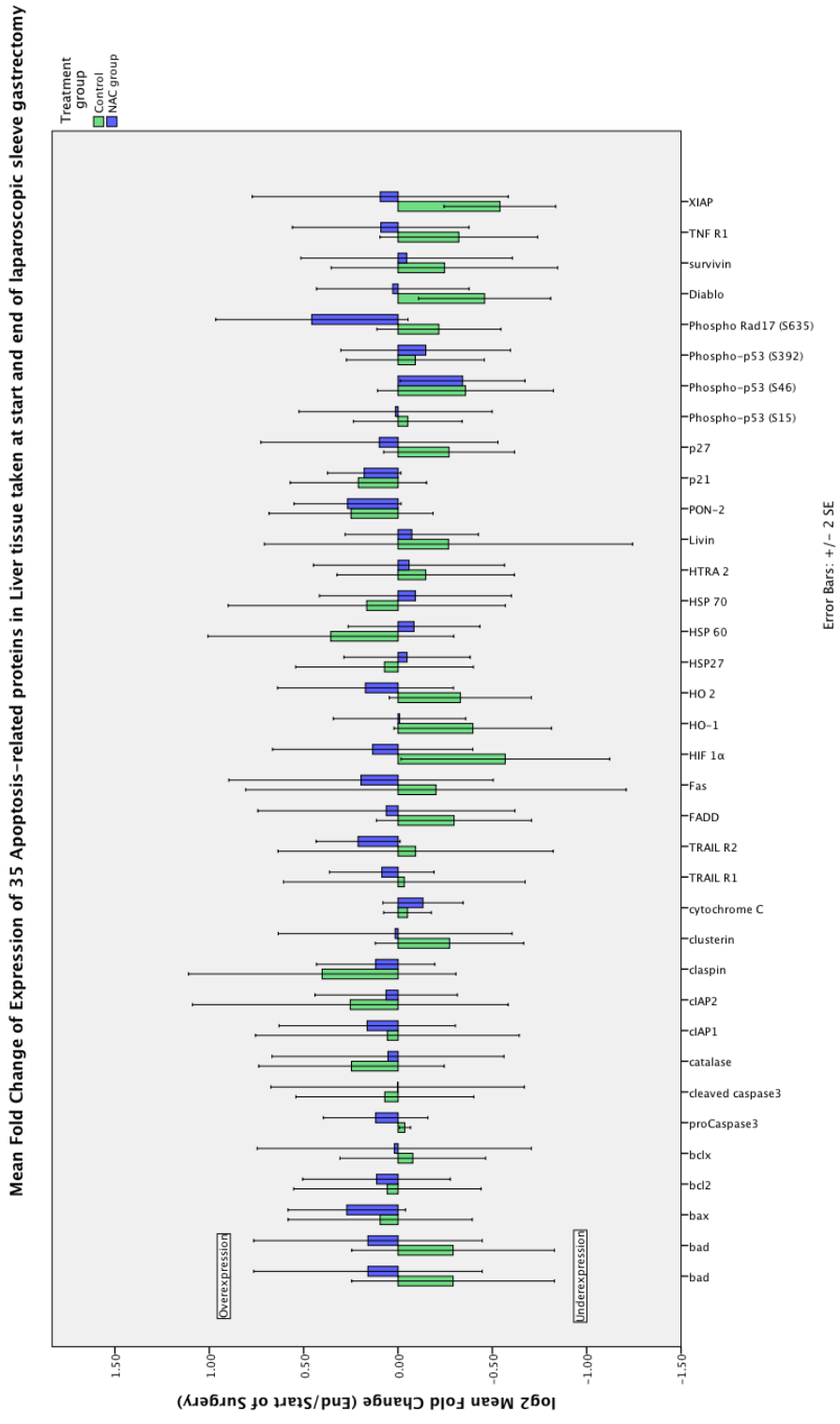
Figure 5.8 CK-18 M30 and M65 levels increase after surgery, but TRAIL and Fas Ligand are not significantly changed.

Values depicted are: CK 18 fragments - mean (95%CI) IU/L; TRAIL and FasL pg/ml. No significant differences between treatment groups but significant increase between day 1 from baseline values (M65 p=0.003, M30 p=0.03).

5.6.3.2 Apoptosis Proteome Profile Array

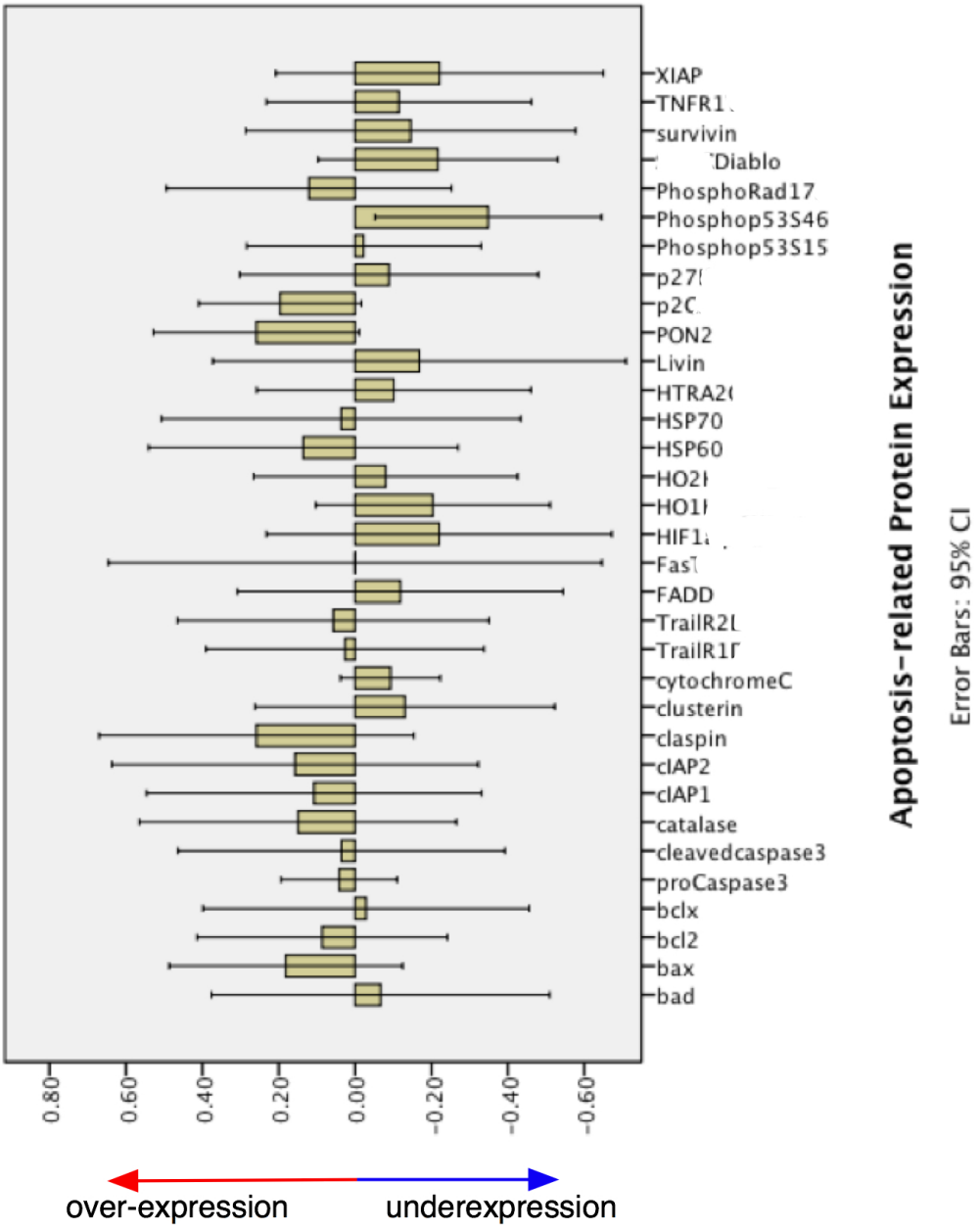
Protein expression was not measured quantitatively using the protein profile array as calibration against a standard curve was not possible. Expression of the 35 measured apoptosis-related proteins is expressed as fold change between start and end of surgery, as defined by the ratio of paired pixel intensities – end/start of surgery. Figure 5.9 shows mean fold change plotted on a log2 scale, showing that the expression profile in the NAC group looks very different to the control group, with more proteins being overexpressed by the end of surgery than in controls. Expression was compared between treatment groups, with independent samples t-tests showing no significant differences. However, the variation in the fold changes is very large (see also error bars on Figure 5.10, which shows combined results for the whole cohort). Mean fold change values for each spot on the proteome profiler are presented in Appendix B1.6 (Table B.7).

Figure 5.9 Fold Change in Expression of 35 Apoptosis-related proteins in both treatment groups



No significant differences between treatment groups (p>0.05). Values are expressed as log2 fold change, where fold change is End/Start corrected pixel density. Abbreviations: cIAP – cellular inhibitor of apoptosis, TRAIL R – TNF related apoptosis inducing ligand Receptor, FADD – Fas associated death domain, HIF – hypoxia inducible factor, HO – haemoxygenase, HSP – heat shock protein, PON – protein paraoxonase, TNF R – tumour necrosis factor Receptor, XIAP – X-linked inhibitor of apoptosis

Figure 5.10 Combined fold changes in apoptosis-related proteins for whole cohort of 14 patients



Note the wide confidence intervals. No comparative statistics possible here.

Values are expressed as log2[fold change], where fold change is End/Start corrected pixel density. Abbreviations: cIAP – cellular inhibitor of apoptosis, TRAIL R – TNF related apoptosis inducing ligand Receptor, FADD – Fas associated death domain, HIF – hypoxia inducible factor, HO – haemoxygenase, HSP – heat shock protein, PON – protein paraoxonase, TNF R – tumour necrosis factor Receptor, XIAP – X-linked inhibitor of apoptosis

5.6.4 Correlations

The data were explored for correlations to evaluate the following relationships:

- Between clinical intraoperative factors and biomarkers
- Between inflammatory markers and measures of oxidative stress
- Between markers of liver injury and markers of inflammation and oxidative stress.

Table 5.2 Correlations between pre-operative factors and biomarkers (Pearson's r)

	Log PEAK CRP FC	Log IL-10 1hr FC	Log IL-6 1hrFC	Log IL-6 24 FC	Log SOD 1hr FC	IL-10 24hr FC	ALT 1hr FC	Log ALT 24hr FC	WCC 1hr FC
Log OpTime	0.098	0.021	-0.093	0.306	-0.108	.495*	0.412	.556*	0.207
BMI pre-op	-0.357	0.053	-0.187	0.107	0.291	0.148	0.261	0.31	-0.423
HOMA pre-op	-0.07	0.158	0.254	0.089	0.203	0.249	-0.079	0.086	0.131
NAS	-0.357	0.311	-0.105	-0.08	0.22	0.054	0.042	0.1	-0.112

*p<0.05. Abbreviations: log – Logarithmic transformation to Base 10, BMI – body mass index, HOMA – measure of insulin resistance, NAS – NAFLD activity score, 1hr FC – fold change between end and start of surgery, 24hr FC – fold change between day 1 and baseline

Increases in post-operative inflammatory and oxidative stress markers did not correlate with the length of surgery, pre-operative BMI or the severity of fatty liver disease. The only exception was a relationship between operating times and IL-10 fold change over the first 24 hours after surgery (Pearson's r=0.495, p=0.044). There was a relationship between SOD and IL-6 (r=0.566, p=0.018) and IL-6 and peak CRP fold changes (r=0.575, p=0.016). In general, there were few significant correlations between inflammatory markers and other markers of injury and oxidative stress.

Table 5.3 Correlations between markers of liver injury and biomarkers of inflammation and oxidative stress

	Log AUC Leptin	Log AUC Adiponectin	Log IL-6 1hr FC	Log I L-6 24 FC	IL-10 24hr FC	Log AUC CRP	CRP 24hr FC	Log ALT 24hr FC
Log SOD 1hr FC	0.161	-0.167	-0.019	.566*	0.127	0.146	-0.132	0.372
Log SOD 24hr FC	-0.06	0.19	-0.057	0.4	0.281	0.059	0.001	.522*
Log M30 24hr FC	0.025	-0.149	-0.285	0.099	0.417	0.407	-0.071	.578*
Log M65 24hr FC	0.118	-0.064	-0.354	0.011	0.089	0.256	-0.332	0.483
Log PEAK CRP FC	-.471*	-0.098	0.334	.575*	0.249	0.634**	.787**	0.012

*p<0.05; **p<0.01. Abbreviations: log – Logarithmic transformation to Base 10, 1hr FC – fold change between end and start of surgery, 24hr FC – fold change between day 1 and baseline

Initial increase in M30 fragments correlates strongly with ALT 24 hour fold-change (r=0.578, p=0.024) and with BMI (r=0.613, p=0.012). There are no correlations with TRAIL and FasL levels.

5.7 Discussion

Laparoscopic bariatric surgery is associated with beneficial health outcomes, including resolution of obesity-related co-morbidities, improved health-related quality of life and prolonged life expectancy. Pneumoperitoneum and intraoperative liver retraction is associated with a number of deleterious findings, inducing an inflammatory response, a rise in transaminases and increased oxidative stress. This occurs due to a combination of pressure-related microcirculatory changes, direct barotrauma to the liver and other viscera and ischaemia-reperfusion injury after the end of surgery.

In chapter 2, the clinical outcomes of this trial were presented, including a significant rise in ALT, AST, WCC and CRP post-operatively. Liver transaminases and WCC rise significantly by the end of surgery, with a peak in ALT and AST on day 1, followed by a return to baseline within 3-4 post-operative days. CRP rise lags behind, peaking on day 2. The many limitations of the study were highlighted, including the observation of wide variations in the extent of derangement of the parameters from patient to patient. NAC administration did not have a significant effect on these clinical parameters. The study was not of sufficient power to comment on the effect of NAC on outcomes, including complications.

In this chapter, the results are presented of a number of interesting laboratory assays undertaken to further investigate post-operative events. Again, the post-operative changes in cytokines, oxidative stress markers and Cytokeratin 18 fragments were subject to a large inter-individual variation. The study has demonstrated that NAC had no statistically significant effect on each of the parameters and did not ameliorate hepatocellular injury. Even so, the data presented here is novel and reveals more evidence regarding the nature of the post-operative response to surgery.

5.7.1 The effect of NAC on oxidative stress

In terms of oxidative stress, indicators of lipid and protein oxidation, TBARS and PC, and GPX, part of the glutathione antioxidant system, did not change significantly after surgery. GPX catalases the conversion of hydrogen peroxide to

water. Hydrogen peroxide is one of the most abundant byproducts of ROS production and leads to lipid and protein peroxidation. GPX is the most abundant antioxidant enzyme within eukaryotic cells, which is the reason this test was selected for this study **(389)**. Ueda *et al* showed that plasma GPX activity had increased by 35% by 1 week after RYGB, accompanied by a significant decrease in adipose tissue levels of 8-*iso*-Prostaglandin F2α (8-*iso*-PGF2α) **(390)**. 8-*iso*-PGF2α is a quantitative marker of tissue oxidative stress, derived from the products of lipid peroxidation. An increase in GPX activity may be interpreted as having a beneficial effect on cell metabolism by reducing ROS-induced damage and knockout mice models corroborate this **(389)**. However there are a number of different GPX isoforms, each with a different gene whose expression is regulated individually **(389)**. The assay used in this study measured total plasma GPX activity and it is possible that expression of the various GPX enzymes is differentially expressed in this cohort. The timescale of the repeated measures of GPX only covered the first post-operative day and induction of GPX may take longer. In myeloid cell lines, Shen *et al* measured expression of cellular GPX mRNA expression and protein levels and supernatant enzymatic activity after induction with phorbol ester (a known stimulant of cell differentiation and inducer of oxidative stress) and demonstrated that increase in GPX levels and activity occurs after 48 to 60 hours **(391)**. NAC is able to increase intracellular glutathione levels but its effect on GPX induction is not clear. In a comparison between non-diabetic and diabetic rats, Ribeiro *et al* found higher levels of GPX activity in the kidney and liver of diabetic rats, but that NAC administration did not increase GPX activity in either group, although NAC was associated with reduced markers of lipid peroxidation **(392)**. This suggests that GPX expression may be associated with diabetes and obesity **(111, 390)** but that its expression is not increased in an acute setting, which contradicts the finding that GPX activity increased within hours of laparoscopic and open cholecystectomy **(369)**.

Both TBARS and PC are indicators of oxidative stress-induced cellular damage and both assays are straightforward enzymatic methods. TBARS is the most widely performed measure of lipid peroxidation. Significant reductions in both parameters have been demonstrated in the medium to long-term after bariatric surgery, indicating the general reduction in oxidative stress **(393)**. Conversely, a

number of studies have shown that malondialdehyde (MDA) levels increase rapidly following surgery. Plasma MDA levels rose rapidly in patients undergoing laparoscopic prostatectomy under general anaesthesia within 60 minutes and 120 minutes of induction of pneumoperitoneum **(394)**. The authors used an MDA ELISA rather than the TBARS enzymatic method used in this study. In a randomised trial of NAC in patients undergoing laparoscopic gynaecological surgery, Beyaz *et al* found that MDA levels (measured by an unspecified method) increased significantly, by 22%, at 1 and 24 hours after surgery in patients receiving NAC, although there was no increase seen in the control group **(395)**. In patients undergoing laparoscopic Nissen fundoplication, which requires liver retraction, TBARS levels rose significantly by the end of surgery and on the first post-operative day. No rise was seen in patients undergoing laparoscopic lower abdominal surgery, such as colectomy **(396)**. A number of studies have demonstrated rise in MDA/TBARS after laparoscopic cholecystectomy. Polat *et al* showed that both TBARS and PC were increased by the end of laparoscopic cholecystectomy **(370)**. Seven *et al* found a rise in MDA and other markers of oxidative stress after both open and laparoscopic cholecystectomy **(369)**. In a similar study, Bukan *et al* showed that TBARS levels rise by more than 50% within 45 minutes of laparotomy incision, with a smaller rise of 12% after induction of pneumoperitoneum **(397)**. Oddly, they found that MDA levels dropped in the laparoscopy group on the first operative day to <50% of baseline values, which they conclude is a beneficial effect of laparoscopy. Tissue MDA levels in small patches of peritoneum rose after both laparoscopic and open surgery **(398)**. Similarly, a number of small animal models of pneumoperitoneum have demonstrated a rise in liver tissue TBARS and PC within hours of surgery **(274, 275, 399-401)**. In contrast, McHoney *et al* measured plasma MDA using high-performance liquid chromatography in children undergoing open and laparoscopic Nissen fundoplication and found no significant changes from baseline at end of surgery or after 4, 24 and 48 hours **(402)**. Aran *et al* measured levels of ischaemia-modified albumin and found an increase in circulating levels within 30 minutes of induction of laparoscopy but did not see a concomitant increase in MDA or other markers of oxidative stress at the same timepoint **(403)**. These results do show very wide confidence intervals, especially at Day 2, suggesting either a very heterogenous insult or problems with sample processing.

SOD levels rose by a factor of mean 4.8 (95%CI 2.7-6.9) by the end of surgery. 1 hour SOD fold change correlates strongly with 24 hour fold change of IL-6, which also correlates strongly with Peak CRP fold change. The inference is that SOD expression is associated with the inflammatory response (as indicated by CRP and IL-6 rises) but that an increase in its expression occurs much sooner than the other parameters. Thus, a rapid rise in SOD levels by the end of surgery predates the other markers of inflammatory response. Interestingly, 24 hour SOD fold change correlated strongly with the 24 hour fold change of ALT, whereas 1 hour fold changes did not. This suggests that transaminase rise and hepatocellular damage is associated with a more prolonged period of oxidative stress, and patients in whom SOD levels return to baseline more rapidly have less hepatocellular damage.

5.7.2 The effect of NAC on cytokines

Interleukin-6 (IL-6) levels rose significantly and peaked by the end of surgery and began to return to baseline on post-operative day 1. IL-6 is the prototypical "pro-inflammatory cytokine" in the literature on post-operative inflammatory response. Bariatric surgery is associated with significant falls in IL-6 over 6-24 months **(90)**. IL-6 rises after laparoscopic and open surgery but the magnitude of rise after laparoscopic surgery is smaller **(404)**. Jacobi *et al* have reviewed studies of laparoscopic versus open cholecystectomy to 2002 and found that 7 studies including 286 patients showed that post-operative IL-6 rise was lower in laparoscopic cholecystectomy than in open, whereas only 1 study of 12 patients showed no difference **(404)**. These types of studies were cited during the introduction of laparoscopic surgery as objective evidence that laparoscopic surgery had certain advantages over open surgery in terms of being a lesser immunological and inflammatory insult **(405)**. IL-6 stimulates production of CRP by the liver as part of the inflammatory response **(91)**. This study confirms this relationship, as 24 hour fold change of IL-6 correlated strongly with peak CRP fold change from baseline (p=0.016).

In this study, IL-10 fold change after surgery was mean 193 (95%CI 37-350). This dramatic increase may reflect the fact that most of the patients were super-obese

(BMI>60kg/m^2), diabetic and had NASH – all factors associated with elevated IL-10 **(406, 407)**. IL-10 has traditionally been viewed as an anti-inflammatory cytokine. Rabelo *et al* found that IL-10 levels correlated with HOMA index and reduced in patients with NASH compared with simple NAFLD **(407)**. Dalmas *et al* have conducted the largest study of bariatric patients to include IL-10 **(406)**. They found that IL-10 was undetectable in the serum of normal weight individuals and was higher in diabetic obese patients than non-diabetic obese patients. After 12 months, IL-10 levels fell in patients undergoing RYGB, along with all other measured cytokines, including IL-6 and CRP **(406)**. NAC has been shown to increase IL-10 levels in a randomised trial of liver transplant patients **(408)**.

Although there was a statistically significant difference in TNFα between treatment groups, this was a spurious finding as is clearly demonstrated in Figure 5.6. Mean baseline TNFα levels were different in NAC and control groups and this difference was maintained across all timepoints. On day 2, TNFα levels were significantly lower than baseline (approximately 50%). In contrast, other studies have shown significant rises in TNFα following laparoscopic surgery, up to 3 fold in gynaecological surgery **(409)**, and in multiple animal models of pneumoperitoneum **(410-412)**. TNFα rises after bariatric surgery have been smaller but statistically significant after laparoscopic gastric banding **(413)** and RYGB **(414)**. Long term studies evaluating TNFα after bariatric surgery have produced conflicting results and a meta-analysis showed that there are no significant changes after 12 months **(90)**.

In one of few studies where inflammatory markers were measured at similar time-points, Abramo *et al* compared the effects of different types of general anaesthesia, xenon, propofol only and sevofluorane, in 33 patients undergoing RYGB **(414)**. They found that the rise in IL-6 levels after surgery and at 12 hours in the three groups were the same order of magnitude as this study: End of surgery fold change from baseline: xenon median 4.9 (range 1.9–11.7), propofol 4.95 (1.5–54.3), sevofluorane 2.7 (−6 to 62.5); 12 hours after 4.55 (2.6–16.5), 5.40 (0.9–61.2), 3.5 (−21.2 to 26.5). Relevant to this study, the fold changes were smallest in the sevofluorane group. Patients in this study were all given sevofluorane anaesthesia, after induction with propofol. They found that IL-10 levels also rose after surgery,

but the mean fold changes were dramatically smaller than I have described, albeit with very wide confidence intervals: Xenon 0.6 (−2.1 to 30.2), propofol 1.85 (−1.4 to 14.3), sevofluorane 5.95 (2.7–18.9) **(414)**. In contrast to IL-6, the largest fold changes occurred in the sevofluorane group. TNFα fold changes are similar to IL-10: Xenon 0.25 (−5.7 to 3.4), propofol 1.45 (−6.4 to 12.9), sevofluorane 3.95 (−3.3 to 8.9).

Chachkhiani *et al* compared cytokine levels after laparoscopic gastric banding and colorectal resection **(413)**. They found that cytokine rises were much smaller in the obese patients undergoing gastric banding than the non-obese colorectal patients. Both groups had lower cytokine levels than controls with proven sepsis. They also found that leptin increased significantly and concluded that it should be considered as an acute phase reactant. Chung *et al* compared the cytokine response to RYGB and laparoscopic gastric cancer surgery **(415)**. They also found much greater rises in the non-obese cancer patients than in the RYGB group (mean BMI 24 vs 43), despite baseline TNFα and IL-6 levels not being significantly different. The gastric cancer patients were significantly older (mean 61 years vs 28). Gastric cancer is known to be associated with increased cytokine levels and surgery is likely to be more complex than RYGB. Despite these confounding factors, the authors postulate that obesity may also be associated with a blunted inflammatory response compared with non-obese patients, despite the presence of a chronic inflammatory state at baseline **(348)**. Contradicting these findings, Di Vita *et al* measured inflammatory markers in obese and non-obese patients undergoing laparoscopic cholecystectomy **(416)**. Compared with non-obese patients, the obese group had significantly higher baseline levels and a greater rise in IL-6 and Leptin 24 hours post-operatively. In contrast, the non-obese group had much higher baseline IL-10 and adiponectin and these levels rose significantly, whilst the obese group had much smaller non-significant rises at 24 hours. They suggest that this data confirms that high levels of IL-10 and adiponectin in non-obese patients dampens the inflammatory response and account for other studies which show an association between obesity and post-operative complications **(417)**.

5.7.3 Acute changes in adipocytokines after laparoscopic bariatric surgery

In this study, the changes in adipocytokines, leptin, resistin and adiponectin, were not significant over the first 2 post-operative days and do not support the existing literature suggesting that these hormones play an important role in the acute phase response. Given that adiponectin and TNFα expression in adipose tissue is inversely correlated **(418)** and that TNFα may control adiponectin expression from adipocytes **(419)**, the findings described here that both adiponectin and TNFα levels fell significantly from baseline on the second post-operative day is difficult to explain. Butner *et al* reviewed 18 studies measuring adiponectin after bariatric surgery and found levels increase by a mean 69.9% after RYGB and 35.8% after restrictive surgery **(361)**.

As discussed in Chapter 1, the rationale for evaluating post-operative adiponectin response was following interest in its potential use as a therapy to ameliorate ischaemia-reperfusion injury in human liver transplantation. Jimenez-Castro *et al* found that steatotic livers had lower levels of adiponectin mRNA expression and administration of adiponectin reduced post-transplantation transaminase rises compared with sham and non-steatotic controls **(196)**. Low levels of adiponectin may facilitate a pro-inflammatory response, whilst exogenous supplementation may decrease oxidative stress, increase free fatty acid clearance (reducing steatosis) and suppress TNFα expression **(195)**. Baseline adiponectin levels in this study did not correlate with post-operative liver injury, as represented by ALT and CK-18 M30 and M65 fragments (including 1 and 24 hour fold changes and area under curve, AUC). The post-operative change in adiponectin, represented by AUC, was strongly associated with the changes in leptin and IL-6 but did not correlate with BMI, NAFLD activity score or operating time. These findings suggest that adiponectin is not an important determinant of liver injury in this cohort. AUC IL-6 is the only cytokine measured in this study that correlates strongly with AUC CRP, confirming its known association with the inflammatory response **(90)**.

5.7.4 CK-18 and apoptosis marker results

Although liver biopsies taken from bariatric surgery patients have been used to evaluate its diagnostic utility in NAFLD/NASH **(420)**, there is only one longitudinal study showing that CK-18 fragment levels fall 6 months after surgery in patients with NASH **(224)**. The data presented above is the first of its kind measuring CK-18 fragments in the first four days after weight loss surgery. Similar to the elevation in transaminases, this study shows that both M30 and M65 fragment concentrations increase dramatically on the first post-operative day, before returning to near baseline by day 4. Although the arithmetic mean of M30 and M65 levels on day 1 are higher in the NAC group, there were no significant differences between treatment groups. Of note, there are very wide confidence intervals on days 1 and 2, suggesting that the extent of injury measured by these caspase-cleaved fragments is very heterogenous. This feature is also reflected in other outcome measures in this study and discussed at length in Chapter 2.

There has also been some interest in the use of M30:M65 ratio as a method of differentiating between modes of cell death, with higher relative levels of M30 reflecting greater apoptosis than necrosis **(421-423)**. However, there are no specific reports specifying the utility of this ratio in any particular disease group, l*et al*one any reports confirming its validity. Previously, the reasons have been discussed that steatotic liver is more susceptible to ischaemia-reperfusion injury and that hepatocellular necrosis is more likely in NASH than in both NAFLD and non-steatotic liver **(181)**. In this study, M30:M60 ratio fell significantly by day 2 from baseline 0.75 (95% CI 0.47 to 1.03) to 0.53 (0.40 to 0.66). Despite a significant increase in M30 on day 1 indicating that apoptotic hepatocyte death does occur, the balance in mode of injury is towards hepatocellular necrosis. Pre-operative starvation for surgery may contribute to this, as shown in mice models where fasting caused depletion of intracellular ATP, limiting the extent of energy-dependent apoptosis **(381)**. These data also show that the rapid elevation and decline in these markers within 2 days of surgery suggest that the liver injury is of a limited duration and no lasting damage is sustained. The initial rise in M30 correlates strongly with ALT and is also strongly associated with BMI. However, there were no other significant associations between CK-18 fragments and other markers or an association with NAFLD activity score.

Given the CK-18 data showing that proportional levels of apoptosis do not increase significantly after surgery, it is not surprising that TRAIL and FasL do not increase. Increased circulating FasL is not significantly associated with NAFLD/NASH **(424)** but is implicated in ischaemia-reperfusion injury. There is also indirect evidence of a potential role for TRAIL with increased expression of TRAIL-death receptor 5 in association with increased apoptosis in steatotic hepatocytes **(425)**. Steatotic livers are more sensitised to FasL and TRAIL mediated apoptosis **(375)**. The significance of the fall in TRAIL on day 1 is not clear. Falls in TRAIL over the longer term may be associated with weight loss and reduction in other markers and this will be explored in the subsequent chapter.

5.7.4.1 Proteome profiler - what does it show?

The proteome profile array was able to give comparative non-quantitative data only, in the form of fold changes. There were no significant changes between treatment groups in the apoptosis profile and there was huge individual variation in protein expression. Despite the profiles of each treatment group looking very different from each other, it is not possible to draw any firm conclusions or even discern any vague patterns. Figure 5.10 presents the aggregated results for all 14 patients included. Again, confidence intervals are wide and there are no similar studies known with which to compare the patterns of mean fold changes.

From the increased expression of bax, bcl2, proCaspase3, cIAP 1 and 2 and reduced expression of extrinsic pathway death receptors, one may loosely infer that the intrinsic mitochondrial pathway of apoptosis may have predominated. It is not clear what effect hepatocyte necrosis would have on the protein levels. Taken together with the concomitant rise in both M30 and M65 levels, the inference is that a process of "necroptosis" occurs following surgery – a combined process of apoptosis and necrosis due to a combination of ischaemia and IRI **(290)**. Future studies would require both larger numbers of samples and confirmatory immunohistochemistry studies to reach clearer conclusions. Proteomic studies in NAFLD have used various mass spectrometry based techniques to generate potential biomarkers and guide future avenues for research **(426)**.

5.7.5 Limitations of this study

The numerous potential flaws in study design have been discussed in Chapter 2. With early stoppage after only 20 participants were recruited, the study is underpowered and therefore the statistical conclusions are at risk of Type II error. The various oxidative stress markers measured here are subject to variations in sample processing and analysis and can be affected by patient factors as well. It was also not possible for financial reasons to perform more assays at all selected timepoints in order to get a more accurate trend in changes over time. Using a larger number of oxidative stress markers or using different markers, such as 4-hydroxynonenal or F2-isoprostanes to measure lipid peroxidation and oxidised amino acids to measure protein oxidation, may have given better results **(366)**. As discussed in Chapter 2, patients were taking their regular medications and some of these may have had an effect. One patient in each group was taking insulin for glycaemic control. Fluctuations in blood glucose levels, with episodes of hyperglycaemia, are associated with increased oxidative stress **(112)**. All patients were given sevofluorane anaesthesia and the effect of this agent on oxidative stress is uncertain. Experiments in animal models show increased levels of oxidative stress after sevofluorane exposure, whilst others have used it as a pre- or post-conditioning agent to reduce oxidative stress **(332, 427)**. The administration of low molecular weight heparin has also been shown to affect TBARS **(428)** and all patients in this study routinely received enoxaparin for venous thromboprophylaxis as per standard practice. Oxidative stress and cytokines may be affected by various concomitant medical conditions, especially diabetes and obstructive sleep apnoea **(429-431)**. In the only published trial of NAC use in laparoscopic surgery, the NAC treatment group had higher levels of MDA and glutathione S-transferase by the end of surgery **(395)**.

5.8 Conclusions

Administration of intraoperative NAC does not significantly reduce oxidative stress, inflammatory response or hepatocyte injury after laparoscopic bariatric surgery. Building on the findings of Chapter 2, the results demonstrate that surgery has a significant but short-lived effect on SOD as a marker of oxidative stress and pro-inflammatory cytokine IL-6, but also causes a large increase in IL-10. Surgery does not have a significant impact on other markers of oxidative stress and the adipocytokines. The mechanism of hepatocellular damage involves both necrosis and apoptosis, confirming the mode of damage is similar to other settings where ischaemia-reperfusion injury occurs and is not mediated by circulating death ligands. Taken together with the findings in Chapter 2, these documented changes in biomarkers are not associated with a significant effect on clinical outcomes. Changes in study design to include a wider range of markers taken at more frequent timepoints in a larger cohort of patients would help to understand the changes further.

CHAPTER 6

THE EFFECT OF LAPAROSCOPIC SLEEVE GASTRECTOMY ON BILE ACIDS, MARKERS OF INFLAMMATION, OXIDATIVE STRESS AND LIVER INJURY AFTER 6 MONTHS

6.1 Introduction

In the previous chapters, the acute inflammatory response after bariatric surgery has been evaluated, including markers specifically looking at liver injury. In contrast, bariatric surgery has beneficial effects in the long term in terms of reducing inflammatory markers and oxidative stress. This is achieved through a combination of weight loss, improved glycaemic control and changes in gut hormone profiles. NASH/NAFLD improves after bariatric surgery, as demonstrated by a number of paired liver biopsy studies showing reduction in steatosis, incidence of NASH and variable improvements in fibrosis. These findings are detailed in Chapter 1-3.

As discussed in Chapter 4, recruitment to the NAC Trial consisted solely of patients undergoing Laparoscopic Sleeve Gastrectomy for logistical reasons rather than through trial design. This presented an opportunity to not only study the effects of bariatric surgery-induced weight loss on markers of liver injury and inflammatory response as planned in the longer term, but also to examine whether LSG had any specific related effects, including on bile acid metabolism.

6.2 Background

6.2.1 Enterohepatic cycling of bile

The development of accurate profiling techniques, such as mass spectrophotometry, and the functional characterisation of orphan receptors, including the nuclear bile receptor farnesoid X receptor-α (FXR), have lead to increased interest in the metabolic and signalling effect of circulating bile acids (BA) **(432)**. Little was known of the more intricate properties of bile beyond its

role in the emulsification and solubilisation of dietary fats to allow efficient digestion and absorption.

Bile production by the liver is the end-stage of cholesterol metabolism and the breakdown of haemoglobin **(433)**. BA are end-products of cholesterol metabolism, formed in hepatocytes. Two primary BA, cholic acid (CA) and chenodeoxycholic acid (CDCA) are conjugated with glycine or taurine. Conjugated BA are excreted into the biliary tree, following concentration of bile in the gallbladder. Solubilisation of ingested dietary lipids occurs in the duodenum and proximal jejunum, leading to the formation of mixed micelles. Micelle formation allows more rapid uptake of fatty acids into enterocytes, from where they are esterified and circulated in the portal blood stream as chylomicrons **(433)**. Up to 50mmol of BA are secreted in to the digestive tract daily, with only approximately 1-5mmol of new BA being synthesised in the liver. Primary conjugated BA are converted to secondary BA by gut microbes, and then can be reabsorbed in the ileum. Thus, over 95% of BA are recirculated to the liver by the portal circulation and can be re-conjugated with glycine and taurine. Previous animal studies have demonstrated that bile exerted a negative feedback effect on cholesterol metabolism and BA production **(432)**.

More recently, *in vitro* and subsequent animal studies have showed that the enterohepatic circulation of BA is regulated at two points in the cycle. Within the liver, cholesterol 7α-hydroxylase (*CYP7A1*) activity is the rate-limiting step in BA synthesis from cholesterol **(434)**. Multiple ligands exert control on *CYP7A1* via the nuclear RXR-FXR, which produces *SHP* protein that binds to the promoter site on the *CYP7A1*. Ileal enterocytes produce fibroblast growth factor 19 (FGF-19) via an FXR-mediated pathway. FGF-19 has a negative feedback on BA synthesis in the liver **(435)**.

Nuclear receptors are transcription factors which can be directly activated by a ligand, either promoting or inhibiting a wide variety of cellular metabolic processes at the gene transcription level. 48 nuclear receptors were identified in humans, including a number of orphan receptors without known ligands. FXR provides a prototypical example of how knowledge of the processes controlling cellular metabolism have been reverse-engineered, in contrast with more

traditional endocrine research, where hormones such as insulin were first identified and purified before downstream targets were identified many years later **(435)**.

FXR was found in high concentration in the liver and intestines and subsequently a large number of ligands for FXR were identified. Wang *et al* demonstrated in a series of *in vitro* cell line experiments that a number of endogenous BA including lithocholic acid (LCA) and chenodeoxycholic acid (CDCA) bind FXR **(436)**. Subsequently Inagaki *et al* demonstrated in a rat model that activation of intestinal

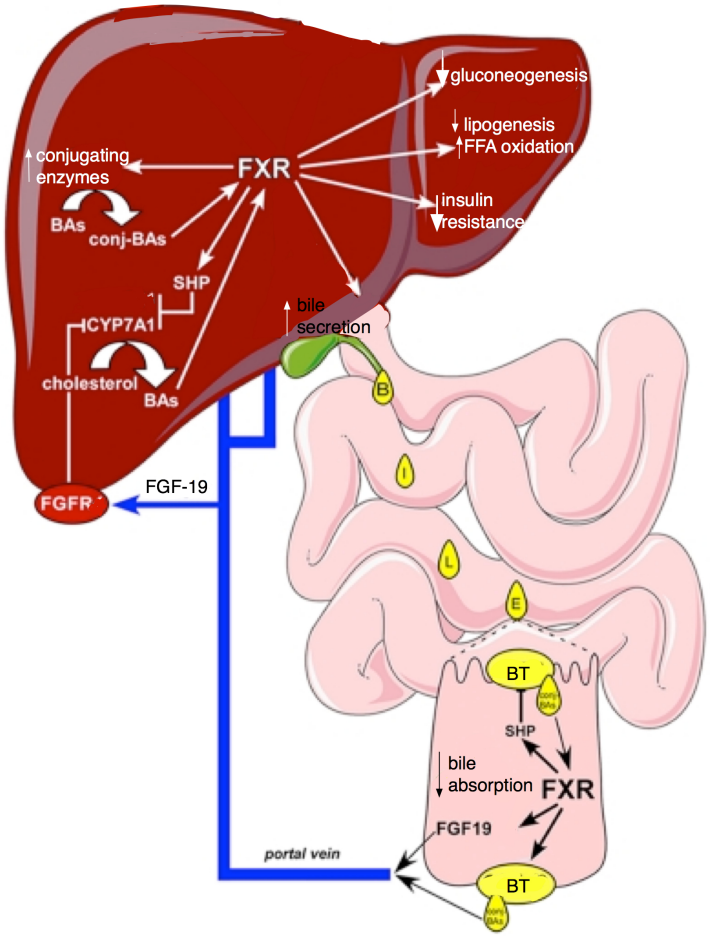


Figure 6.1 The enterohepatic circulation of bile is controlled by FGF-19, via FXR (adapted from Modica *et al*, 2010) (432)

Fibroblast growth factor (FGF-19) production in the terminal ileum is stimulated by bile acids (BA) and has pleiotropic effects on the liver via the Farnesoid X receptor (FXR) and the FGF receptor (FGFR). The synthesis of BA from cholesterol is inhibited by *SHP* via suppression of *CYP7A1* activity, the rate-limiting step in BA synthesis. FXR activation also leads to increased conjugation and excretion of BA from the liver. It is now recognised that FXR has a number of metabolic effects, decreasing insulin resistance and gluconeogenesis and increasing lipid clearance and free fatty acid (FFA) oxidation in the liver. In the terminal ileum, FXR reduces expression of bile transport

(BT) proteins, leading to increased faecal loss of BA and reduction in the total BA pool size.
FXR by BA lead to the production of fibroblast growth factor 15 (FGF-15), the rat homolog of FGF-19 and that this in turn inhibited cholesterol synthesis in the liver by repression of *CYP7A1* **(434)**. Figure 6.1 depicts this process.

6.2.2 A wider role for BA, FXR and FGF-19

Recent years have seen an exponential interest in the potential role of bile beyond merely emulsification and solubilisation of dietary fats to allow efficient digestion and absorption. The identification of the FXR transcription pathway has in turn lead to characterisation of a number of other related receptor pathways, including liver orphan receptor (LXR), retinoid X receptor (RXR), pregnane X receptor (PXR) and other BA regulating genes, including *CYP7B1* and *CYP27A1* **(437)**. There are a large number of individual BA that act as ligands of FXR, as well as other intracellular compounds, including various cholesterol moieties and arachidonic acid metabolites. Many of these potential ligands have been identified as having an action at supraphysiological doses and it is unclear if they have a significant or specific role *in vivo*. The role of FXR has now extended beyond cholesterol regulation to include lipoprotein, glucose metabolism and liver regeneration. Aberrant FXR function is also implicated in carcinogenesis and chronic liver diseases. Loss of regulation via FXR can lead to high circulating BA, which may cause liver toxicity **(438)**.

BA also act through TGR5, a G-protein coupled receptor found in enteroendocrine cells, in high concentrations in brown adipose tissue, stomach, pancreas and liver **(439)**. Via a cAMP-dependent pathway, TGR5 activation is associated with an increase in GLP-1. Thomas *et al* were able to demonstrate that TGR5 activation by a synthetic agonist lead to calcium dependent release of GLP-1 from a cultured enteroendocrine cell line **(440)**. They then used the same agonist in various knockout and diabetic mice models to show that TGR5 activation leads to improved glucose homeostasis and decreased insulin resistance, associated with increased GLP-1. TGR5 may also suppress production of inflammatory cytokines **(51)**. TGR5 is also linked to resting energy expenditure, by promoting

the activation of intracellular thyroid hormone. Watanabe *et al* were able to reduce weight gain in a high-fed mouse model by supplementing their diet with Cholic Acid CA, leading to higher energy expenditure as measured by indirect calorimetry and also decreased accumulation of white adipose tissue **(441)**. TGR5 is present in the liver and in TGR5 knockout mice, total BA pool size is reduced by ≤25%, indicating some involvement in BA homeostasis **(439)**.

6.2.3 FXR, FGF-19, Bile Acids and Glucose regulation

Following binding with ligand, nuclear receptors such as FXR are able to initiate transcription of the target genes via their DNA-binding domain. These receptors act as sensors within the cell milieu and able to modulate cell processes in response to any changes. Transgenic mice experiments provided much of the early evidence for FXR. FXR knockout mice have increased insulin resistance. Administration of a synthetic FXR agonist to diabetic mice lowered blood glucose and increased hepatic glycogen production, whilst overexpression of FXR also produced the same changes **(442)**. FXR activation also increased intestinal FGF-19 and FGF-21 production. Exogenous FGF-19 caused weight loss and improved insulin sensitivity in another mouse model.

Clinical studies have also demonstrated that the FXR-FGF-BA axis is important in humans. Administration of the bile acid sequestrant colesevelam leads to improved glucose control in Type II diabetics (T2DM) whose existing glucose control was poor, in a number of large scale placebo-controlled studies of patients taking either metformin or insulin-based therapy **(443)**. This improvement is through the changes in circulating BA profile, mediating effects via the FXR pathway **(444)**.

6.2.4 Bile acids and Fatty liver disease

As discussed in Chapter 2, the crucial events in the pathogenesis of NAFLD/NASH are the accumulation of intra-hepatocyte lipid, formation of damaging Reactive Oxygen Species due to mitochondrial fatty acid oxidation and dysfunction and activation of a local inflammatory response, eventually leading to hepatocyte

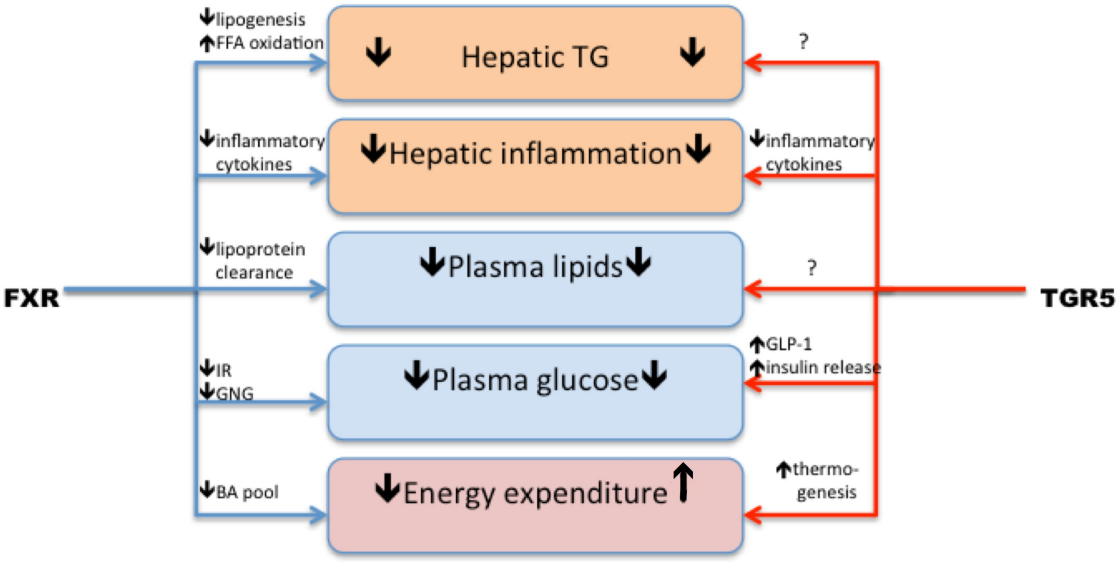
apoptosis and necrosis. These activities also stimulate fibrosis. This process occurs in the context of hepatic insulin resistance. Both FXR and TGR5 play important roles in regulating lipid metabolism and hepatic triglyceride (TG) synthesis, as well as modulating production of inflammatory cytokines in liver macrophages, monocytes and Kuppfer cells **(51)**. FXR knockout mice have elevated plasma TG levels, hepatic steatosis and elevated levels of TNFα and other pro-inflammatory cytokines. Studies involving TGR5 knockout mice suggest that it also mediates anti-steatotic and anti-inflammatory effects **(439)**. The range of effects of FXR and TGR5 activation on metabolism are summarised in Figure 6.2. There is ongoing clinical interest in the use of exogenous BA for the treatment of NAFLD/NASH. Ursodeoxycholic acid (UDCA) is a secondary BA that has been shown to exert an anti-apoptotic effect on hepatocytes, reduce circulating TNFα levels and improve hepatic insulin sensitivity **(445)**. A systematic review of 12 studies of UDCA in 1160 patients has shown that treatment with oral UDCA leads to reduction in ALT and reduced fibrosis and steatosis **(446)**. Methodological quality of these studies is poor and open to risk of bias so UDCA cannot be unequivocally recommended as a treatment. Nevertheless, there is much interest in the concept. Recently, a synthetic version of CDCA, obeticholic acid (OBCA) has been developed. OBCA has a 100-fold greater affinity to the FXR receptor and a number of cell line and animal studies have shown that it can exert anti-inflammatory effect in the liver, reduce hepatic fatty acid synthesis and improve hepatic insulin sensitivity **(447)**. A phase II clinical trial of a 6 week course of OBCA in patients with T2DM and NAFLD showed improvements in insulin sensitivity and reduced non-invasive markers of hepatic fibrosis in the treatment groups **(448)**.

6.2.5 Bile Acids and Bariatric Surgery

With increasing interest in the role of BA in diabetes and introduction of bile sequestrants as treatment for T2DM, it is no surprise that there are a number of studies looking at the effects of bariatric surgery on BA profile. As detailed in Chapter 1, bariatric surgery leads to dramatic improvement in insulin resistance and glycaemic control. After RYGB, the improvement in IR occurs within days of

surgery before any significant weight loss occurs. A number of groups have studied both immediate and long-term changes in BA after bariatric surgery.

Of 10 published studies including patients undergoing bariatric surgery, nine studies included patients undergoing RYGB (see Appendix 3 Table C.1 for a summary of these studies) **(449-458)**. All nine studies show that total BA increase following RYGB surgery, although the timing of the increase is variable. In the largest cohort study, Gerhard and colleagues included 186 patients undergoing RYGB (BMI>35) and also included 110 overweight controls (BMI 30-35) **(450)**. This was a cross-sectional study with RYGB patients included at variable timepoints following surgery (median 140-150 days). In patients who had not been operated, diabetic patients had lower circulating FGF19 and higher total BA compared with patients without diabetes. Although total BA and FGF19 levels were lower in obese patients who went on to have surgery, the differences were not significant and there was no correlation when the whole group was analysed together. There was an inverse correlation between FGF19 and hepatic *CYP7A1* expression. In patients post-RYGB, FGF19 and total BA increased non-significantly. Subgroup analysis showed that the increases were significantly higher in patients



whose diabetes went into remission. The design of the study and the variable timing of the post-RYGB samples make it difficult to draw definitive conclusions. The results

Figure 6.2 The pleiotropic effects of bile acid receptors FXR and TGR5 on hepatic and whole body metabolism, glycaemic control and insulin resistance (adapted from Li *et al*, 2013) (51)

FXR improves insulin resistance, reduces inflammation and decreases energy expenditure. The interaction with the effects of TGR5 is not fully elucidated but it appears that TGR5 has mainly effects on improving insulin resistance and increasing energy expenditure. FXR agonists are already in clinical use and there is increasing interest in harnessing the effects of TGR5 for clinical use.

suggest that diabetes is associated with lower FGF19 and BA levels and that these rise post-RYGB, especially in diabetic patients whose glucose control improves. Most pertinently, even in this relatively large study, the variability of FGF19 and BA levels is high, with standard deviations often exceeding the given mean value.

Dynamic measurement of the post-prandial BA response was performed in two smaller studies by Ahmad *et al* (5 patients with RYGB) and Kohli *et al* (8 RYGB) **(449, 453)**. Ahmad *et al* show that the post-prandial rise in total BA is smaller in obese patients compared with non-obese controls. Post-RYGB, both fasting and post-prandial BA rise is greater. Kohli *et al* similarly demonstrate a significant increase in the post-prandial BA rise compared with the pre-operative values. Ahmad *et al* do not specifically look at the role of diabetes and Kohli *et al* excluded diabetic patients. Pournaras *et al* showed that the rise in total BA and FGF19 occur at 4 days and 42 days, before significant weight loss has occurred **(456)**.

The overall inferences to be drawn from these studies are that RYGB leads to increases in FGF (4 studies) and BA, either fasting and/or post-prandial **(450, 452, 455, 456)**. Given the dramatic and rapid reduction in insulin resistance after RYGB, one might postulate that the FGF-BA axis is implicated in this improvement.

In contrast, the effect of LSG on enterohepatic circulation has not been studied in depth. Nakatani *et al* included 15 (/34) patients who underwent restrictive surgery and had an undefined mixed group including both LSG and LAGB **(454)**. LAGB has been shown to have no significant effect on circulating total BA in two other studies with a total of 18 LAGB patients (Kohli *et al*, 2013 and Pournaras *et al* 2012). However Nakatani *et al* report dramatic rises in BA in the restrictive group

that exceed the levels seen in their malabsorptive group of patients undergoing RYGB and BPD. Steinert *et al* report no significant difference in total BA over the first year after LSG in 7 patients, compared with the RYGB group who only had significant increases at the 12 month timepoint **(458)**. Haluzikova *et al* included 17 non-diabetic females undergoing LSG and compared them with 15 healthy controls **(451)**. They report a significant increase in FGF19 after 6 months but no significant changes in total BA.

6.3 Aims of this chapter

The aim of this study is to evaluate the effects of LSG at 6 months post-surgery on markers of inflammation, oxidative stress and liver injury. Given the interest in the effects of bile acids on fatty liver disease, the aim was to determine if there is any relationship between weight-loss related changes in insulin resistance, lipid profiles and circulating BA levels. There is a paucity of research into the effects of LSG on BA metabolism and the findings of this study will further aid the understanding of these changes.

6.4 Methods

6.4.1 Patients and Samples

Patients were recruited as part of the NAC Trial. Protocol details are given in **Section 4.3**.

Fasting serial serum and plasma samples were taken before surgery (prefix "Pre-") and 6 months after surgery (suffix "-6mon"). Liver tissue samples were taken at the beginning of surgery for histopathological analysis. Details are given in **Section 4.3.3.3.**

6.4.2 Routine Assays performed

The following assays were performed as part of routine blood biochemistry and haematology testing on an automated multi-analyser, the Advia 2400 (Siemens Healthcare Diagnostics, Camberley, Surrey, UK) in the Departments of Biochemistry and Haematology at King's College Hospital: ALT, AST, CRP, glycated haemoglobin (HBA1c), full lipid profile (total cholesterol, triglycerides, low-density lipoprotein (LDL), high-density lipoprotein (HDL), non-esterified fatty acids (NEFA), fasting insulin and fasting glucose.

Insulin Resistance was measured by calculating HOMA-IR as

$$\frac{\text{Fasting glucose (mmol/l) x fasting insulin (mU/l)}}{22.5}$$ **(319)**.

6.4.3 Biomarker assessment

The following assays were performed at pre-operative and 6 month timepoints:

- TBARS (see Section 5.5.1.1 for Laboratory methods)
- GPX (see Section 5.5.1.4)
- IL-6, IL-10, TNFα, Leptin, Resistin, Adiponectin (see Section 5.5.2.1)
- Cytokeratin M30 and M65 (see Section 5.5.3.1)
- TRAIL and FasLigand (see Section 5.5.3.2).

6.4.3.1 Plasma Fibroblast Growth Factor 19

FGF-19 was measured using Quantikine Human FGF-19 Immunoassay from R&D Systems Europe Ltd (Abingdon, UK). This is another sandwich enzyme immunoassay and the protocol is exactly the same as that given in Chapter 3 Section 3.4.3.2 for TRAIL and FasL. The only substitutions are Calibrator Diluent RD5P is used instead. FGF-19 standards used were of the same concentrations as described in the TRAIL and FasL ELISAs.

6.4.3.2 Hyaluronic Acid

The TECO Hyaluronic Acid ELISA kit (TECOmedical AG, Sissach, Switzerland) was used to measure HA. This is sandwich enzyme immunoassay, using a horseradish peroxidase (HRP) conjugated HA binding protein (HABP). This is coated on the microplates and catalyses a substrate reaction in a quantitative manner.

Sample timepoints used for HA assay were Pre-operative and 6 months.

6.4.3.2.1 Reagents and Standards used in the assay

HA binding protein-coated 96 well plate
HABP-HRP conjugate
6 premixed HA Standards: 0, 15, 50, 150, 450 and 1000 ng/ml
Sample diluent
Tetramethylbenzidine Solution in two parts - stabilised TMB and stabilized hydrogen peroxide
Wash Buffer – buffered surfactant with preservatives

Stop Solution – sulphuric acid 1M

Deionised water

Standards came in separate prefilled vials. Each standard was diluted 1:50 with sample diluent in a ratio of 10µl Standard:490µ diluent. 100µl of each standard was transferred to the 96 well plate in duplicate.

6.4.3.2.2 Reagent and Sample Preparation

All reagents were brought to room temperature before use. Wash and Cell Lysis buffer concentrates were diluted with deionised water.

Serum sample aliquots were thawed on ice. 1:50 sample dilution was performed by mixing 10µl of sample in 490µl of Sample Diluent. 100µl of diluted sample per well were transferred to the ELISA plates in duplicate, according to a predetermined plate map.

6.4.3.2.3 Assay procedure

100 µl of sample and standard was added to each well, in duplicate according to a plate map. The covered microplate was incubated for 2 hours at room temperature, placed on a horizontal shaker set at 500 rpm.

At the end of this period, the wells were aspirated and washed three times, with a volume of 350µl Wash Buffer per well per wash. After the last wash, the plates were inverted forcefully onto blotting paper to ensure all Wash Buffer had been removed. 100 µl of the relevant HABP-HRP was added to each well. The plates were again incubated for 30 hours at room temperature, on a shaker as before. The washing step was repeated five times as above. 100 µl of the relevant TMB-Substrate solution was added to each well. Both plates were mixed gently and incubated for 30 minutes at room temperature, in the dark, on a shaker set at 500rpm.

At the end of this period, 100 µl Stop Solution was added to each well and the plates were gently shaken to ensure mixing.

The absorbance was read by a microplate reader at 450 nm with the reference filter set between 590-650nm (Dynex MRX, Dynex technologies Ltd. Worthing,

UK). The standard curves were generated, using Linear regression with Log/Log axes scaling. R^2 was 0.99 for both plates. Concentrations of HA were interpolated from the standard curves.

6.4.4 Bile acids by LC-MS/MS

BA were measured by Dr Royce Vincent, Consultant in Clinical Biochemistry at King's College Hospital in the Department of Biochemistry. He kindly provided the protocol described below.

Sample timepoints used for BA analysis were pre-operative and 6 months.

6.4.4.1 Reagents and Standards used in the assay

All solvents and chemicals were sourced from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, UK) unless otherwise stated.

Helix pomatia sulphatase enzyme
Sodium acetate
Sodium ascorbate
Formic acid
Phosphate-buffered saline (PBS)
Methanol
Acetonitrile and ammonium acetate (Rathburn Chemicals Ltd, Walkerburn, Scotland)

Sodium salts of DCA, GDCA, TDCA, CDCA, GCDC, TCDC, UDC, TUDC, LCA, GLCA, TLCA, CA, GCA and TCA
GUDC (Merck Serona UK, Feltham, UK)
Deuterated (d) internal standards deoxycholic-2,2,4,4-d4 acid (d4-DC) and glycodeoxycholic-2,2,4,4-d4 acid (d4-GDC) (QMX Laboratories, Essex, UK)
Taurodeoxycholic-2,2,4,4-d4 acid (d4-TDC) synthesised in-house (Zhang *et al*, 1993)

6.4.4.2 Sample Preparation

Extraction of BA from EDTA-plasma was performed as described previously by Tagliacozzi *et al* **(459)**. A protein precipitating solution of acetonitrile containing each of the internal standards; a 2,2,4,4,d4- DC for each of the BA chemistries: unconjugated, glycine- and taurine- conjugated was prepared. 800µL of protein precipitating solution was added to 250µL of sample and vortex-mixed. Samples were centrifuged at 13000 RPM for 10 minutes. 900µL of the supernatant was transferred into fresh microtubes. The supernatant was then blown to dryness using compressed air in a 60°C heated block. The residue was re-constituted in 250µL of a 50:50 (v/v) mix of mobile phases A and B. The re-constituted solution was transferred to HPLC vials and 10µL injected onto the column.

6.4.4.3 Assay Procedure

BA fractions were analysed using an in-house method on a HPLC system composed of the following: three PU-2080 analytical pumps, MX-2080-32 solvent mixing module, AS-2059 auto-sampler and CO-2067 column oven (Jasco™, Tokyo, Japan). The HPLC system was connected to an API3200TM triple quadruple mass spectrometer (Applied Biosystems®, Life Technologies Ltd, Paisley, UK) operated with an electrospray ionisation source. Chromatography was performed using an Acentis fused-core C18 analytical column (150x4.6mm, particle size 2.7µm; Sigma-Aldrich, Dorset, UK) incubated at 40°C. Mobile phases comprised (A) methanol or (B) deionised water each containing 5 mM ammonium acetate and 0.012% formic acid. Negative ion mass spectra of the fractionated BAs were recorded in multiple reactions monitoring (MRM) mode. Data were acquired using provided ChromPass software (Jasco™) and quantitated using peak area analysis corrected by comparison to the respective internal standard. The method was linear between 0.1 and 10 µmol/L for all BAs and their conjugates. An example chromatogram of the BA fractions is shown in Figure 6.3.

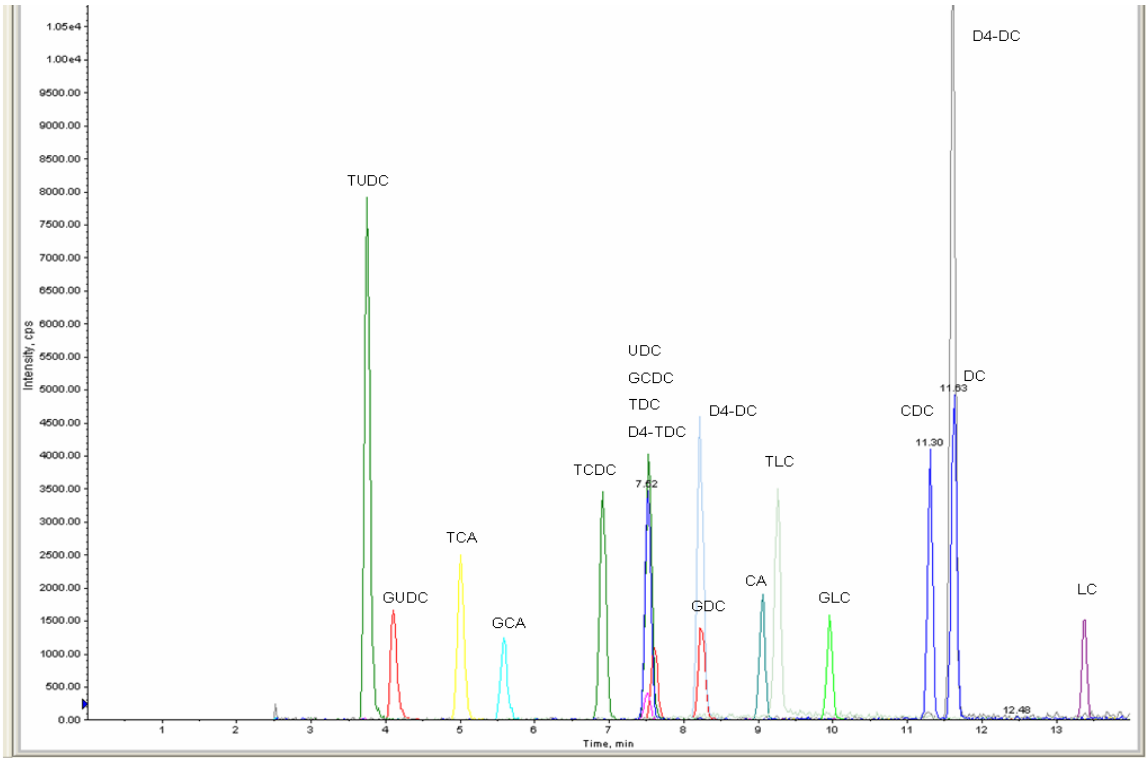


Figure 6.3 Example Chromatogram of BA fractions
(from left to right, TUDC-Tauroursodeoxycholic acid, GUDC-Glycoursodeoxycholic acid, TCA-Taurocholic acid, GCA- Glycocholic acid, TCDC-Taurochenodeoxycholic acid, UDC-Ursodeoxycholic acid, GCDC-Glycochenodeoxycholic acid, TDC-Taurodeoxycholic acid, D4-TDC-Taurodeoxycholic-2,2,4,4-d4 acid, D4-DC-Deoxycholic-2,2,4,4-d4 acid, GDC-Glycodeoxycholic acid, CA-Cholic acid, TLC-Taurolithocholic acid, GLC-Glycolithocholic acid, CDC-Chenodeoxycholic acid, DC- Deoxycholic acid, LC- Lithocholic acid).

6.4.5 Statistical Analysis

Statistical analyses were performed using SPSS 20 (SPSS, USA).

Before comparative statistical tests were performed, outliers were sought visually using boxplots. The assumption of normality was tested for each parameter, using values of the difference between paired samples before and 6 months after surgery using the Shapiro-Wilk's test ($p>0.05$). Only BMI, ALT and AST met the criteria of having minimal numbers of outliers and normally distributed paired differences. These data are therefore presented as Mean with 95% confidence intervals and paired t-tests were performed to compare the two timepoints. All other parameters are presented as median ± interquartile range and Mann-Whitney-U tests were performed to compare timepoints. For ease of comparison, all continuous data were presented graphically as boxplots, including BMI, ALT and AST. Note that the median values for these three parameters were almost the same as mean values.

For parameters whose values changed significantly between timepoints, a fold change ratio was calculated as [6month value]/[Baseline value]. These values were log2 transformed. The assumption of normality was tested using Shapiro-Wilk's test ($p>0.05$). Linearity was checked visually using scatterplots. Pearson's correlation was performed between these parameters to look for any significant associations. The sample size was too small to perform regression analysis.

Clinical significance was determined if $p<0.05$.

6.5 Results

6.5.1 Clinical parameters and Biomarkers

Of 19 patients who entered the NAC trial, 18 patients completed 6 months follow up. Basic demographic data and information about co-morbidities are given in Chapter 2. After six months, patients lost a mean 47kg (95%CI 30 – 64), which represents mean percentage excess body weight loss (%EBWL) 42% (95%CI 36 – 49) over six months. Liver transaminase levels were all within the normal range at outset and both ALT and AST fell significantly. Markers of glycaemic control and insulin resistance all significantly improved (see Table 6.1). Seven of 8 patients with an abnormal pre-operative HBA1c >6.5% experienced a reduction of HBA1c to the normal range. Seven of 9 patients without a pre-operative diagnosis of diabetes mellitus had a fasting blood glucose (FBG) > 5.6 mmol/L before surgery. This is the International Diabetes Federation definition of impaired fasting glucose or "pre-diabetes", and at 6 months, only 1 patient still met this criterion. All 15 patients with obstructive sleep apnoea experienced significant improvement in their symptoms and only 3 were still using their continuous positive airway pressure (CPAP) machines.

Pre-operative lipid profiles were all in the normal range as all eleven patients with dyslipidemia were being treated with lipid-lowering medications. Non-esterified fatty acid levels fell significantly after 6 months (see Table 6.1 and Figure 6.4).

Markers of oxidative stress, TBARS and GPX, did not change significantly over the study period. Leptin concentration fell significantly, but Resistin and Adiponectin did not change over the study period. Pro-inflammatory markers, IL-6 and CRP, fell significantly over the study period (see Figure 6.5)

CK-18 fragments fell significantly, but TRAIL and FasL levels did not change significantly (see Figure 6.6).

Table 6.1 Changes in clinical and biomarker values at baseline and 6 months after Laparoscopic Sleeve Gastrectomy

	Before LSG	After 6months	p value
Weight kg	167 (155 – 182)	121 (104 – 139)	<0.001
BMI kg/m^2	60.6 (55.3-65.8)	45.8 (41.5 - 50.1)	<0.001
ALT IU/L	31 (26 - 37)	18 (15 - 22)	<0.001
AST IU/L	27 (23 - 30)	20 (17 - 23)	<0.001
Fasting Glucose mmol/L	7.2 (6 - 8.8)	4.8 (4.4 - 5.7)	0.004
Fasting Insulin mIU/L	25 (14.2 - 33.8)	8.6 (5.5 - 13.4)	0.001
HOMA-IR	7.7 (5.3 - 11.8)	1.7 (1.2 - 3.1)	0.001
HBA1$_c$ %	6.5 (5.9 - 7.7)	5.5 (5.1 - 6.1)	0.008
Cholesterol mmol/L	4 (3.7 - 4.6)	4 (3.6 - 4.7)	ns
Triglycerides mmol/L	1.6 (1.1 - 2.2)	1.3 (0.9 - 1.7)	ns
LDL mmol/L	2.2 (1.7 - 2.7)	2.4 (1.8 - 3.3)	ns
HDL mmol/L	1.1 (1 - 1.2)	1.1 (0.9 - 1.2)	ns
NEFA mmol/L	0.7 (0.48 - 0.93)	0.45 (0.37 - 0.56)	0.008
TBARS nmol/ml	14.4 (6.6 - 24.7)	13.8 (5.7 - 16.6)	ns
GPX U/ml	8.9 (4 - 11.9)	10.8 (8.8 - 12.9)	ns
CRP mg/L	10.3 (6.8 - 20.7)	6 (5 - 17.4)	0.05
IL-6 pg/ml	6 (3.5 - 8.3)	4.3 (3.2 - 5.5)	0.04
IL-10 pg/ml	0.7 (0.3 - 1.1)	0.7 (0.7 - 0.7)	ns
TNFα pg/ml	7 (4.1 - 7.6)	7.3 (6.2 - 9.4)	ns
Leptin ng/ml	142 (101 – 206)	48 (20 - 84)	0.001
Resistin ng/ml	8.5 (6.1 – 15.2)	9.9 (6.2 – 13.8)	ns
Adiponectin ng/ml	9285 (6956 - 11917)	9760 (6403 - 12154)	ns
HA µg/L	30.7 (20.1 - 53.5)	25.8 (11.9 - 63.8)	ns
M65 IU/L	249 (202 - 535)	240 (163 - 264)	0.015
M30 IU/L	174 (123 - 251)	132 (105 - 140)	0.001
CK18ratio	0.64 (0.36 - 0.89)	0.55 (0.45 - 0.67)	ns
TRAIL pg/ml	100.6 (65.3 - 120.3)	95.4 (81.6 - 116.4)	ns
FasLigand pg/ml	94.6 (63.4 - 107.1)	84.3 (80.2 - 112.1)	ns
AST/ALT ratio	0.9 (0.8 - 1)	1 (0.9 - 1.4)	0.005

ns – non significant (p>0.05)

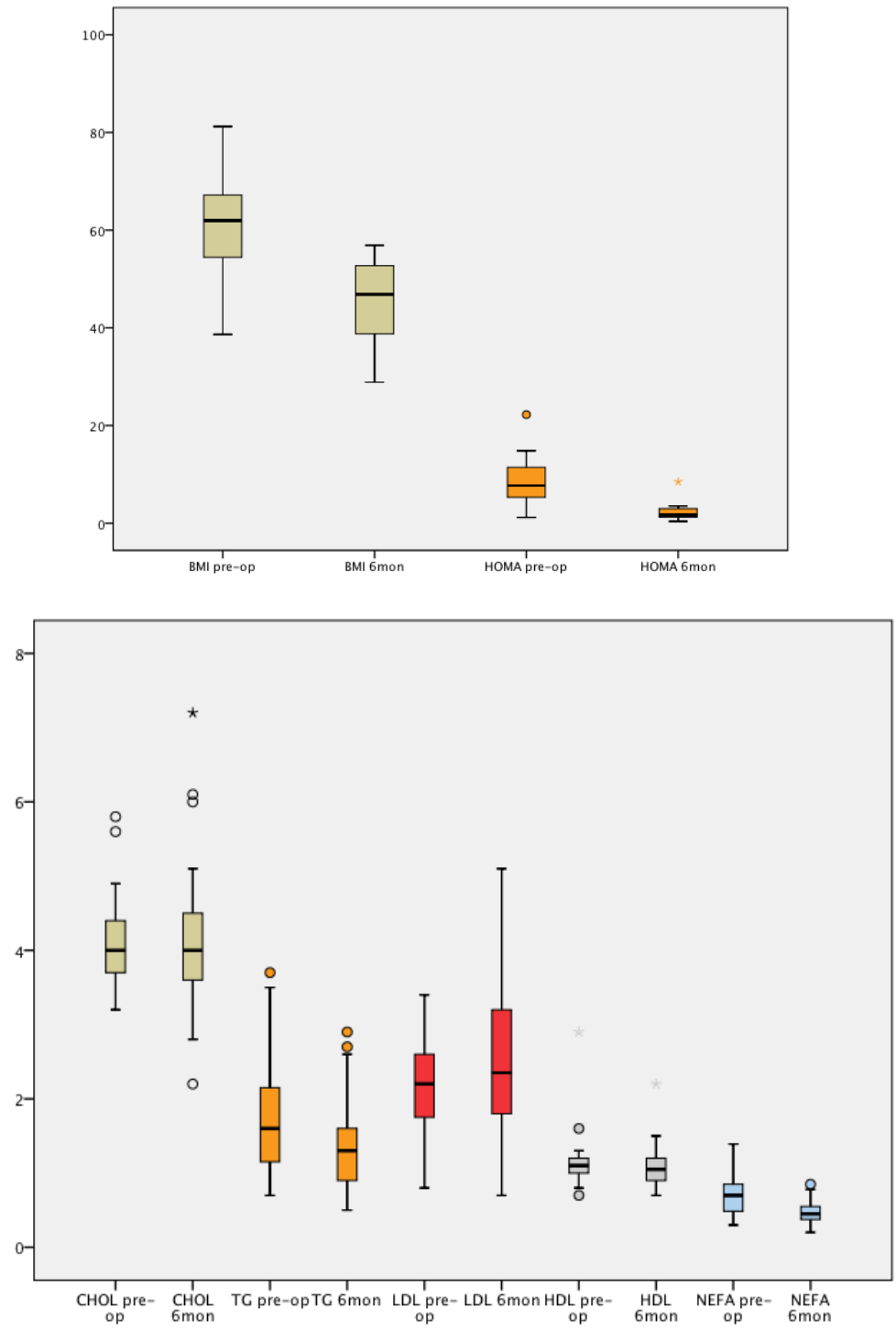


Figure 6.4 Boxplots BMI, HOMA-IR and Lipid profile at baseline and 6 months after LSG

Abbreviations and Units: Body mass index (BMI) kg/m^2, Lipids all mmol/L; Chol - Total cholesterol, TG – triglycerides, LDL – low density lipoprotein, HDL – high density lipoprotein, NEFA – non-esterified (free) fatty acids. BMI, HOMA-IR and NEFA values fell significantly over 6 months (see Table 4.1)

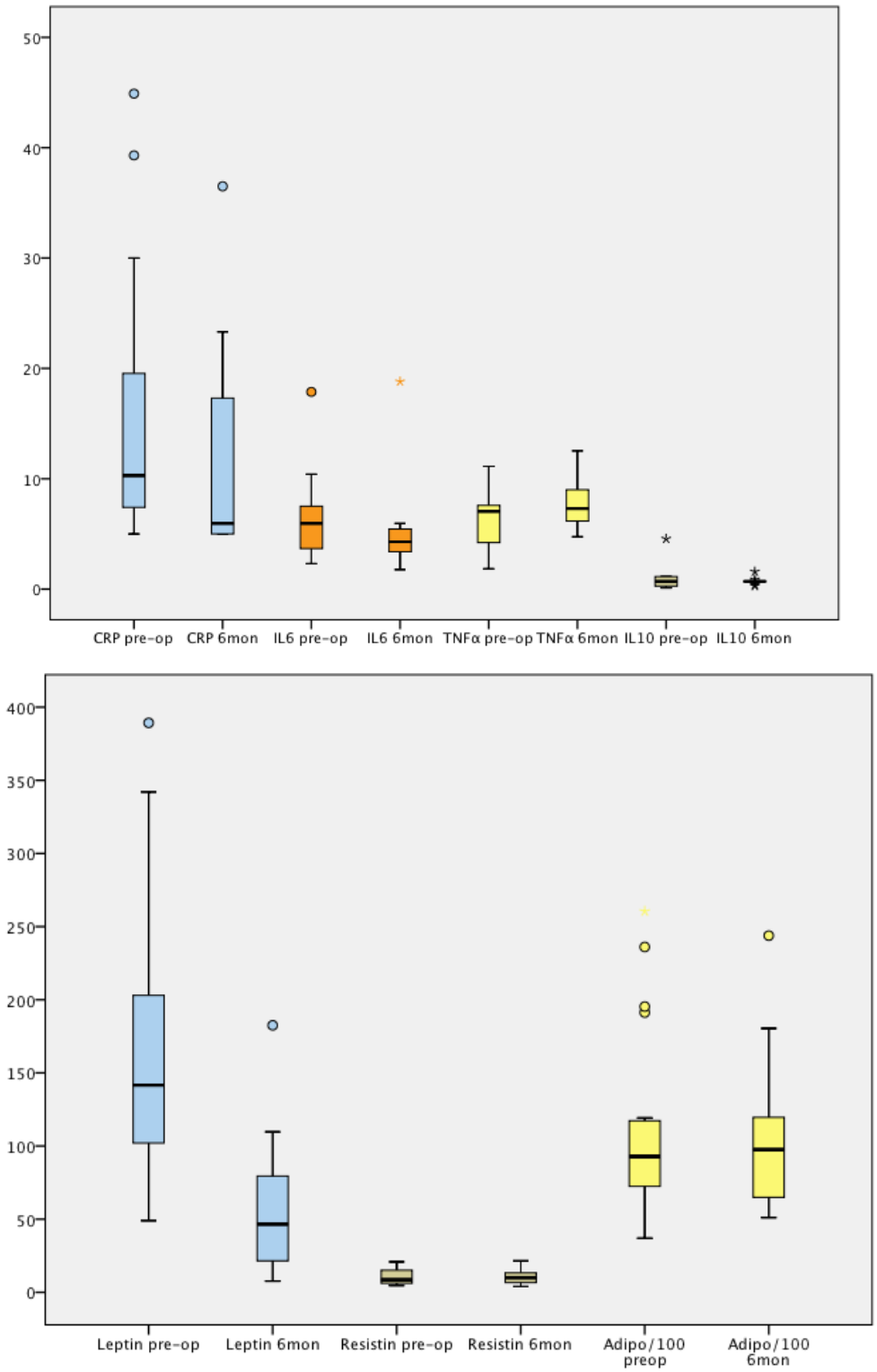


Figure 6.5 Boxplots of CRP, Cytokines and Adipokines at baseline and 6months
Abbreviations and Units: C-reactive protein (CRP) – mg/L; Interleukin 6(IL6), Interleukin 10 (IL10), Tumour Necrosis Factor (TNF)α – all pg/ml; Leptin, Resistin – ng/ml; Adiponectin values are $x10^{-2}$ to keep it on the same scale – ng/ml

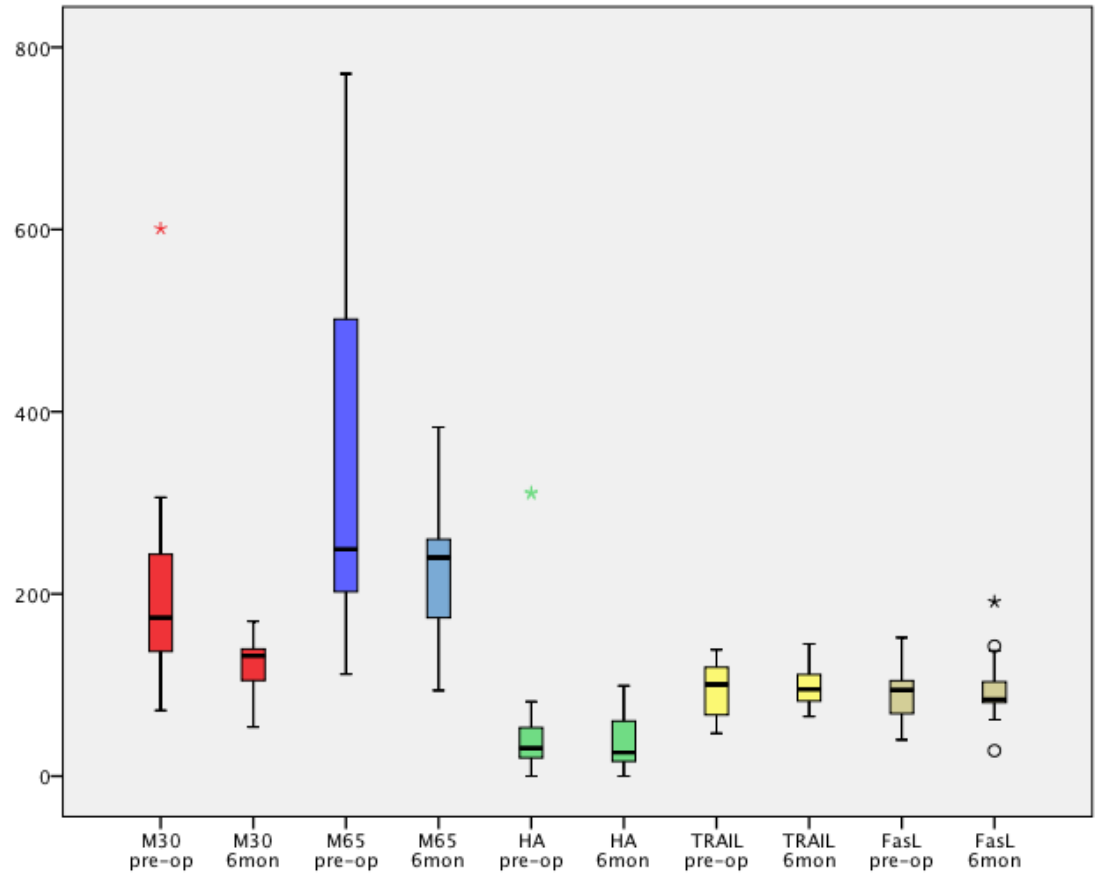


Figure 6.6 Boxplots of CK-18 fragments M30 and M65, Hyaluronic Acid, TRAIL and FasLigand

Abbreviations and Units: M30 and M65 – IU/L, Hyaluronic Acid (HA) - µg/L, TRAIL and Fas Ligand – pg/ml. M30 and M60 fall significantly over 6 months ($p<0.01$)

6.5.2 FGF-19 and Bile Acids

FGF-19 levels rose significantly over the study period, from a baseline median 128.1 (89.4 - 210.1) to 177.1(121.8 - 288.9, p=0.045). This was accompanied by no significant changes in total bile acid concentration. More detailed analysis of specific constituent bile acids showed significant rise in glycoursodeoxycholic acid (GUDCA) and falls in glycocholic acid (GCA), deoxycholic acid (DCA) and taurocholic acid (TCA) (see Table 6.2). Fold change values were calculated as ratios of 6 month: Baseline concentrations and represented in Figure 6.7.

Table 6.2: Bile acid profiles before and 6 months after LSG surgery

Bile Acid profiles	Before LSG	6 months after LSG	p value
Fasting BAs (µmol/L)			
Total BA	2.35 (1.41 - 3.66)	2.41 (1.84 - 3.04)	ns
Total GCBA	1.14 (0.67 - 1.96)	1.49 (0.73 - 2.14)	ns
Total TCBA	0.24 (0.16 - 0.36)	0.17 (0.14 - 0.32)	ns
Total unconjugated BA	0.58 (0.41 - 0.95)	0.46 (0.25 - 0.81)	ns
Primary BAs			
CA	0.06 (0.02 - 0.2)	0.03 (0.03 - 0.08)	ns
CDCA	0.14 (0.08 - 0.31)	0.09 (0.05 - 0.23)	ns
Primary conjugated BAs			
GCA	0.2 (0.08 - 0.3)	0.09 (0.04 - 0.17)	0.048*
TCA	0.01 (0 - 0.07)	0 (0 - 0.02)	0.039*
GCDC	0.51 (0.22 - 1.21)	0.56 (0.25 - 1.12)	ns
TCDC	0.09 (0.04 - 0.15)	0.03 (0.02 - 0.14)	ns
Secondary BAs			
DCA	0.24 (0.16 – 0.42)	0.13 (0.09 – 0.23)	0.048*
UDCA	0.06 (0.04 - 0.07)	0.07 (0.03 - 0.32)	ns
LCA	0.01 (0.01 - 0.02)	0.01 (0.01 - 0.02)	ns
Secondary conjugated BAs			
GDCA	0.19 (0.12 – 0.36)	0.15 (0.10 – 0.37)	ns
TDCA	0.08 (0.05 - 0.11)	0.07 (0.05 - 0.08)	ns
GUDCA	0.1 (0.05 - 0.15)	0.26 (0.06 - 0.63)	0.004*
TUDCA	<LOD	<LOD	
GLCA	0.07 (0.06 - 0.08)	0.07 (0.06 - 0.08)	ns
TLCA	0.06 (0.06 - 0.06)	0.06 (0.06 - 0.07)	ns

Abbreviations: RYGB, Roux-en-Y gastric bypass; BAs, Bile acids; CA, CDCA, DCA, LCA, UDCA, cholic, chenodeoxycholic, deoxycholic, lithocholic and ursodeoxycholic acids, respectively; G, T indicates glycine or taurine conjugation; LOD, limit of detection. Values are expressed as median with inter-quartile ranges (IQR). P value was obtained by Mann-Whitney U test. ns – non-significant

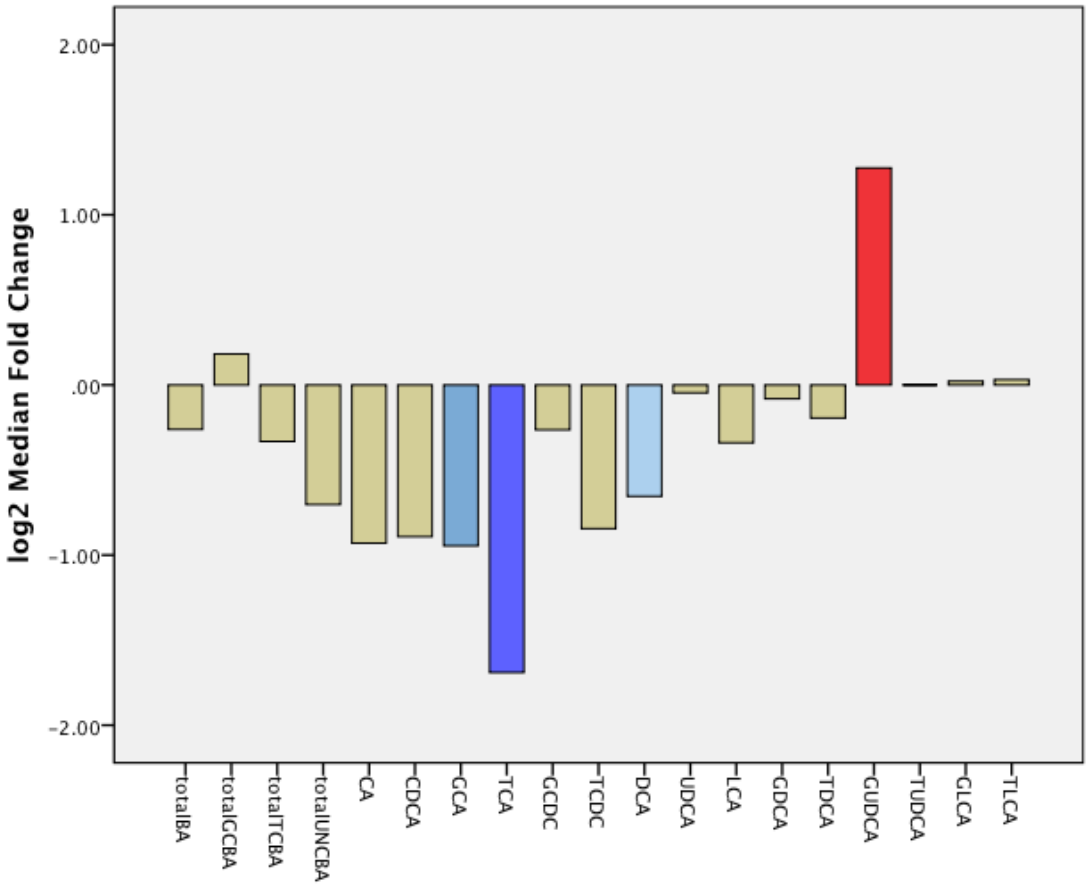


Figure 6.7 Bile Acid Profile represented as Median Fold Change 6month/Pre-op values on a log 2 scale

Abbreviations: RYGB, Roux-en-Y gastric bypass; BAs, Bile acids; CA, CDCA, DCA, LCA, UDCA, cholic, chenodeoxycholic, deoxycholic, lithocholic and ursodeoxycholic acids, respectively; G, T indicates glycine or taurine conjugation;

6.5.3 Correlations

Correlations between the parameters were explored in an attempt to define these relationships:

- between weight and pre-operative insulin resistance and inflammatory markers and/or oxidative stress
- between markers of liver injury and inflammatory markers/oxidative stress
- whether the parameters that changed significantly over the study period were related to individual changes in bile acids.

Pre-operative BMI correlated with CRP (Spearman's rho=0.596, p=0.007) and HOMA correlated significantly with baseline Resistin (rho= 0.574, p=0.02). There was no correlation with other inflammatory markers or oxidative stress.

Post-operative BMI correlated with IL-6 (rho=0.554, p=0.04) and 6 month HOMA still correlated significantly with Resistin (rho=0.846, p<0.001).

Pre and post-operative inflammatory markers do not correlate significantly with TBARS or GPX.

Pre-operative M30 correlates with GPX (rho=0.467, p=0.044), and negatively with Leptin (rho=-0.481, p=0.037) and TRAIL (rho=-0.496, p=0.031). At 6 months, M30 correlates only with TNFα (rho=0.546, p=0.043).

The levels of four bile acids changed significantly over the study period (see Table 6.2). The fall in GCA correlated significantly with the fall in M30 (p=0.003, see Figure 6.8). The fall in TCA correlated with the fall in Leptin (p=0.04) and the fall in DCA was associated with a fall in fasting insulin (p=0.019). There was no correlation between a rise in FGF-19 levels and bile acid concentrations, nor with HOMA or inflammatory markers. The fall in GCA and fall in M30 levels correlated significantly (after log2 transformation, Pearson's r 0.700, p=0.003, see Figure 6.8).

Table 6.3 Correlations between changing BA and biomarkers – Pearson's r

	FGF19	BMI	Insulin	HOMA	NEFA	IL-6	CRP	Leptin	M30	M65
GCA	0.21	0.21	0.45	0.47	0.26	0.14	-0.05	0.48	.70**	0.32
TCA	0.56	0.31	0.56	0.59	0.46	-0.34	-0.52	.68*	0.63	0.14
DCA	0.27	0.02	.60*	0.42	-0.31	0.48	-0.08	0.24	0.41	.50*
GUDCA	0.11	-0.28	0.11	0.09	-0.40	-0.06	0.20	-0.07	0.30	0.35

All parameters transformed log [base 2]. *p<0.05, **p<0.01

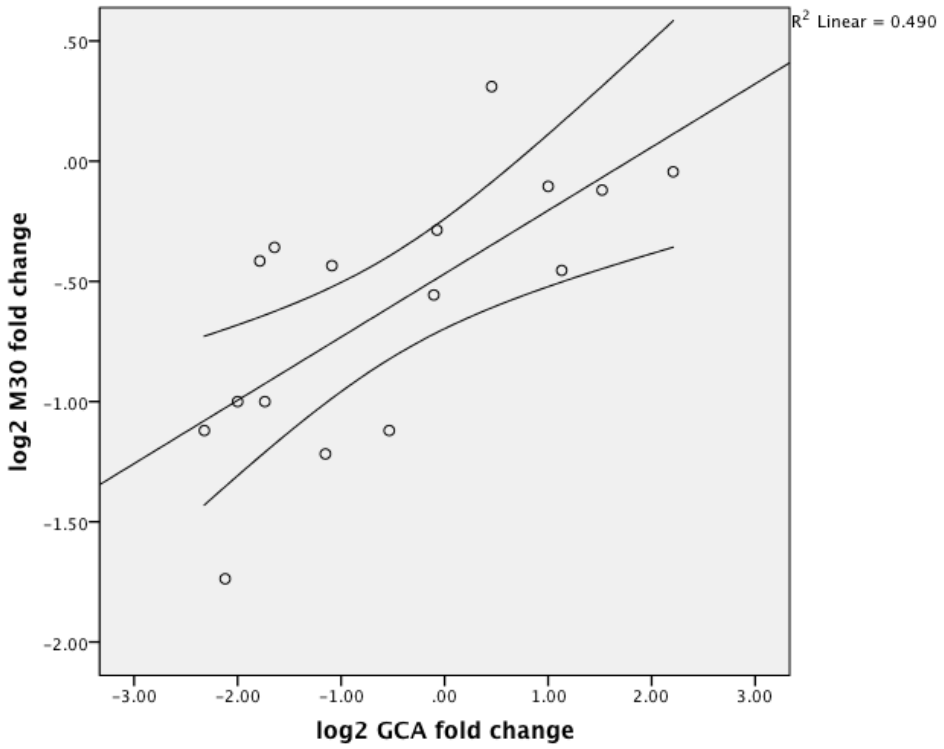


Figure 6.8 Scatterplot of log2 GCA and log2 M30 depicted significant correlation (p=0.003)

6.6 Discussion

6.6.1 Clinical results

This study demonstrates that LSG is associated with significant weight loss over the first 6 months, along with dramatic improvements in insulin resistance, HBA1c and reduction in co-morbidities. Mean %EBWL of 42% concords with the published literature **(36)**. Diabetes mellitus improved in 9 of 10 patients and only 3 of 15 patients with OSA still required CPAP machines at the end of the study. In a meta-analysis looking at diabetes resolution after LSG, Yip *et al* reported that resolution rates (as defined by $HBA1_c$<6.5%) were 56% at 3 months, 68% at 1 year and 80% at 36% after SG. This study confirms the findings of other centres that LSG offers comparable short and medium-term results to RYGB.

Lipid profiles did not change significantly over 6 months and this reflects the aggressive pre-operative treatment of the study participants with lipid-lowering medications. Perioperative statin use is associated with a lower risk of cardiovascular events in patients undergoing cardiac and non-cardiac vascular surgery and is recommended for use in all high-risk patients **(460)**. The patients in this cohort were at higher risk of complications due to their multiple co-morbidities, including diabetes mellitus, hypertension and OSA, and the extent of their morbid obesity (mean BMI>60kg/m^2) **(461)**. Only two patients had a total cholesterol >5mmol/L, both of whom had no previous diagnosis of dyslipidemia and were not on lipid-lowering medications. All patients on statin medications had pre-operative cholesterol <5mmol/l (mean 3.9). Similarly, it was not possible to show any relationship between TG levels and severity of NAFLD (as measured by NAS) as concentrations were within normal range before and after surgery **(462)**.

NEFA levels fell significantly in the study cohort. NEFA or free fatty acids (FFA) are released into the plasma by breakdown of adipocyte triglycerides (TG) and are taken up in other organs either to synthesis TG or be used as an energy source **(463)**. NEFA levels are higher in obese and diabetic subjects although the relationship is complex and not linear **(463)**. Decrease in NEFA are associated with improvements in insulin resistance, reflecting decreased lipolysis in adipose tissue, improved peripheral glucose utilisation and changes in whole-body energy

metabolism **(464)**. The fall in NEFA levels do not correlate with changes in biomarkers or bile acid profile in this study. However, there is much interest in post-prandial NEFA levels in T2DM, with studies showing that post-bariatric surgery falls in circulating NEFA correspond to resolution of diabetes **(465-468)**. There are no published studies evaluating NEFA post-LSG. Kawano *et al* have studied FFA concentrations after SG and GB in an obese rat model and showed that FFA is significantly lower after SG than GB **(469)**. Although there are no comparative RYGB data in the present study, the results presented above are novel and add to the growing literature regarding the metabolic impact of LSG **(56)**. Further studies including evaluation of the incretin effect, along with post-prandial changes, are required to elucidate the mechanisms of this metabolic impact.

6.6.2 Oxidative stress after LSG

As discussed at length in the Introduction, obesity is associated with increased markers of oxidative stress and bariatric surgery is associated with reduction **(70)**. Levels of TBARS, measuring MDA as a marker of lipid peroxidation, and GPX, used here as a measure of antioxidant defense, did not change significantly after 6 months. This is in contrast to a number of studies, as described in Chapter 1 Section 1.6.4. Cabrera *et al* measured a decrease in MDA from 16.7 to 9.11 nmol/g protein 12 months after RYGB, a 45% reduction **(110)**. Uzun *et al* recorded a 22-24% reduction in TBARS 6 months after open and laparoscopic adjustable gastric banding **(108)**. GPX has not been studied previously in bariatric surgery patients over a 6 month time period, although it is the most abundant cytosolic antioxidant **(389)** and an increase within 1 week of RYGB has been measured **(390)**. The results here may be due to the participants having very high BMIs, so that at 6 months, mean BMI was still >45 kg/m2. This indicates these patients were still morbidly obese and still subject to the pathophysiological sequelae of obesity. The patients in the studies quoted above had baseline BMI of 47 and 53-54, falling to 30 and 40-41 respectively **(108, 110)**. Boesing *et al* found that TBARS levels increased dramatically by 84% 6 months after RYGB, although levels of all antioxidants also increased. Myeloperoxidase, an enzyme secreted by activated neutrophils that generates ROS and causes lipid peroxidation, decreased significantly by 40% over the same time period **(113)**. The authors' conclude that lipid peroxidation may persist for some time after surgery, despite weight loss.

Taken together with the findings presented here, the inferences are that TBARS and GPX either do not accurately reflect the state of oxidative stress or that they both measure a particular end-product that is specifically not changed after surgically-induced weight loss.

There were no demonstrable correlations between oxidative stress and glycaemic parameters. Although there is convincing evidence that diabetes is associated with oxidative stress, many of these studies looked at tissue markers **(68)** and direct relationships with blood glucose, HOMA or HBA1c have not been demonstrated elsewhere either **(110)**.

6.6.3 Cytokines

In keeping with much of the published literature, these results show that LSG had a measurable impact on inflammatory markers, reducing CRP and IL-6 significantly **(90)**. TNFα levels did not change significantly and this reflects the findings of multiple other studies **(90)**. This may reflect limitations of the use of circulating TNFα as a marker of inflammation, as other studies have shown decreased adipose tissue expression and decreased circulating levels of TNF receptors, as discussed in Chapter 1 Section 1.6.3.1 **(93, 470)**. IL-10 levels are not significantly different. Although it has anti-inflammatory effects in animal models and *in vitro*, the relationship between IL-10, obesity and NAFLD/NASH is not clearly elucidated **(351)**. Dalmas *et al* reported a biphasic change in IL-10 after RYGB, with a fall to ~33% of baseline at 3 months, return to approximate baseline levels at 6 months and then a further fall to ~66% baseline at 1 year **(406)**.
Levels of pro-inflammatory cytokines correlate with BMI and confirm the relationship between increasing obesity and inflammation **(68)**.

There were no strong correlations between inflammatory markers and oxidative stress. The majority of similar published studies have focused on one or other and no previous direct relationships have been shown. Nevertheless, there is strong *in vitro* and animal model evidence of the relationship between obesity, inflammation and oxidative stress, as discussed at length in Chapter 1. Although this study does not clearly demonstrate this relationship in the context of weight loss, it seems

reasonable to assume this may reflect limitations of the study. These limitations are discussed further below.

6.6.4 Adipocytokines

Leptin levels fell by >50% over the six months of this study and this echoes the results of other studies showing a fall after bariatric surgery and relationship to adipose mass **(101)**. However, many other authors report corresponding increases in adiponectin associated with weight loss **(361)**. These results show no significant changes, despite comparable weight loss to other published studies. As discussed above, the lack of change in adiponectin may reflect the continuing extent of obesity in this patient cohort and there is certainly no evidence to suggest leptin and adiponectin levels are inversely correlated. Weight loss may exert differential effects on each of their levels. Butner *et al* found a significant correlation between decrease in BMI and increase in adiponectin levels in a meta-analysis of 18 studies **(361)**. Such a finding would imply that the results here might reflect a Type II statistical error (that is, adiponectin levels do rise after surgery but this study fails to demonstrate this). Arvaniti *et al* found that adiponectin levels were lower in patients with biopsy-proven NASH compared with NAFLD, and slightly lower in patients with normal ALT and AST, and did not correlate with BMI or HOMA **(471)**. In the cohort of patients studied here, the majority of patients had NASH rather than simple steatosis, as well as normal liver function and these factors may be important confounders.

Although resistin does not change significantly after LSG, there is a significant correlation to both pre- and post-operative HOMA. This association has been demonstrated previously and may reflect a causative relationship between resistin and hepatic insulin resistance **(364, 472)**. Resistin has been found to increase after RYGB and LSG **(365)** and may be associated with NASH **(363)**.

6.6.5 Liver injury and Apoptosis after LSG

Although a number of studies using serial liver biopsies after bariatric surgery have demonstrated improvement in NAFLD/NASH, the significant reduction in CK-18 fragments described adds to the growing weight of evidence of its usefulness as a marker of fatty liver disease **(221)**. Only two other studies have serially

measured CK-18 levels after bariatric surgery. Diab *et al* reported a median M30 level of 226U/L (IQR 177-298), which fell a median 44% after 6months **(223)**. Kahraman *et al* also report a significant fall in both M30 and M65 after various types of bariatric surgery **(224)**. In Chapter 3, a significant correlation between acute post-operative ALT and rises in CK-18 M30 was demonstrated. M30 also correlates significantly with NAS, confirming its utility as a marker of NAFLD severity as shown by others **(223)**. CK-18 M30/M65 ratio does not change significantly over 6 months. Taken together with the lack of changes in TRAIL and FasL levels, the inference is that the improvement in obesity-induced liver injury does not occur through a particular change in the levels of necrosis and apoptosis. These data favour a concept of global amelioration of these functions rather than one specific mechanism. This is supported by a lack of any strong associations of CK-18 M30 or M65 fold changes with particular changes in individual cytokines. Although pre-operative M30 correlates with GPX and Leptin, confirming that the pre-existing liver injury is associated with oxidative stress and inflammation, these relationships are not maintained after weight loss.

6.6.6 Bile acids and their potential metabolic effects after LSG

Total BA concentration does not change significantly after LSG whilst FGF-19 levels do rise significantly, by a median increase of 51% from baseline, over 6 months. These findings echo Haluzikova *et al*, who recorded approximate increase from 100pg/ml to ~180 pg/ml (estimated visually from graph) in 6 months **(451)**. They found that FGF-19 levels approximated those in non-obese controls 6 months after surgery in a cohort of non-diabetic women. This study differs substantially in that a mixed cohort of men and women, with and without diabetes were studied, without any independently evaluated non-obese controls. Nevertheless, the concentrations presented above are within the same range of values. BA stimulates FGF-19 release in the terminal ileum, which then acts as negative feedback on hepatic BA synthesis. After RYGB, FGF-19 levels also increase and this is associated with an increase in total BA **(450, 473)**. In one RYGB study, a control group of AGB patients demonstrated no changes in FGF-19 or BA **(456)**. The explanation of why increases in FGF-19 after both RYGB and LSG are not associated with a corresponding decrease in total BA, as would be assumed given

the role of FGF-19 as a negative feedback inhibitor of hepatic BA synthesis, is not clear.

The proposed mechanism for the increase in FGF-19 is by increased delivery of unmixed BA to the terminal ileum after the anatomical changes in RYGB. These anatomical changes are not seen after LSG. However, a number of studies have shown that gastric empyting and intestinal transit are both increased after LSG **(474-476)**. The increase in FGF-19 is accompanied by an increase in incretin release and postulated to be a possible mechanism for improvements in post-RYGB diabetes mellitus **(473)**. Small animal surgical models in which nutrient and bile delivery to the terminal ileum is expedited anatomically confirm this incretin effect **(477)**. Chambers *et al* have also shown in a rat model of SG that direct nutrient delivery into the duodenum in rats with SG is associated with an increased release of GLP-1 compared with sham animals. This increase is comparable to RYGB rats and SG rats taking nutrients orally and indicates that SG induces intestinal adaptations and hormonal alterations outwith the effect of anatomical bypass **(478)**. Similar studies have not yet been performed in humans.

In this study, two primary conjugated BA, TCA and GCA levels fell significantly along with secondary BA DCA. The secondary BA GUDCA was increased significantly. Taken together, these changes may be due to altered BA conjugation/deconjugation metabolism by gut microbiota. Gut microbiota have been shown to change after RYGB, with heterogenous changes in the variety of bacterial species, which are associated with changes in the expression of inflammation-associated genes in adipose tissue **(479)**. This clinical study confirms multiple animal model experiments that have shown an association with weight loss surgery, changes in gut microbiota and alterations in BA profile **(480, 481)**. Part of the mechanism of these microbiota-induced changes is due to alterations in circulating lipopolysaccharides (LPS). LPS, also known as bacterial endotoxins, are structural components within gut microbiota and are known to exert an immune response **(482)**. This low-grade inflammation is also implicated in the development of insulin resistance and other components of the metabolic syndrome, including NASH **(483, 484)**. Monte *et al* have demonstrated reduction

in circulating LPS after RYGB, associated with improvements in insulin resistance **(117)**.

The strong correlations between falls in TCA and Leptin and DCA and fasting insulin highlight the myriad possibilities of individual BA as signalling molecules. DCA is a ligand for both FXR and TGR5, both of which are involved in glucose and lipid metabolism (see Figure 4.2) **(485)**. TCA has been shown to downregulate *CYP7A1* transcription in a model of rat hepatocytes **(486)**. Although the role of *CYP7A1* is mainly recognised as the rate-limiting step in BA synthesis, increased hepatic *CYP7A1* expression was also associated with lower FGF-19 levels in diabetic patients undergoing RYGB **(450)**. Falling leptin levels are associated with reductions in pro-inflammatory cytokines and it is thus possible to make a speculative inference that changes in BA profile are responsible, in this case a fall in TCA. The relationships demonstrated in this study give rise to many potential avenues of further research into the potential effects on glycaemic control and metabolism of BA.

6.6.7 BA, LSG and the effect on fatty liver disease

Along with the potential effect on inflammation and insulin resistance, there is great interest in BA as modulators of NAFLD/NASH, including the clinical use of exogenous BA or synthetic derivatives as FXR ligands **(446, 448)**. The fall in GCA correlates with reduction in post-operative CK-18 M30 levels. Although there is little other supporting evidence for a direct role of GCA in inducing NASH from either *in vitro* or animal studies, there are studies suggesting GCA is involved in liver injury. Luo *et al* looked at patterns of BA fractions in various mouse models of drug-induced liver injury and found differentially altered CA, GCA and TCA levels associated with particular patterns of injury **(487)**. In particular, they found increased levels of conjugated primary BA after induction of necrosis and bile duct hyperplasia. Zhang *et al* found increased urinary GCA in patients with hepatocellular carcinoma and used bioinfomatic tools to generate potential pathways by which GCA could be implicated in carcinogenesis in the liver **(488)**. In NASH, CA, DCA and CDCA were found in elevated concentrations compared with controls and CA levels correlated with histological grading of inflammation **(489)**. Of note in this study, the authors did not specifically profile GCA, TCA or any secondary conjugated BA.

The most statistically significant change in BA profile was the increase in GUDCA after LSG. Given the interest and substantial experimental evidence for ursodeoxycholic acid (UDCA) as a potential treatment for NASH, it is especially pertinent to consider the importance of this finding, as GUDCA is the active metabolite of UDCA after glycine conjugation. In a mouse hepatocyte model, UDCA administration was associated with a hepato-protective effect, inhibiting apoptosis **(445)**. The authors of this study concluded that this protective effect was mediated by alteration of the intracellular FFA pool by shifting metabolism from saturated to monounsaturated fatty acids. In both high-fat diet steatotic mice and a NASH mouse model, Pathil *et al* showed that intraperitoneal injections of UDCA ameliorated signs of liver injury and altered lipid metabolism **(490)**. Similarly, Buko *et al* found that UDCA reduced liver steatosis and inflammatory markers in a rat model of NASH **(491)**.

Finally, Bechmann *et al* investigated BA profiles in obese patients with NAFLD/NASH and found that serum BA was higher in NASH than simple steatosis, as was CK-18 M30 **(492)**. They showed that BA inversely correlated with adiponectin. They also showed that both adiponectin and FFA upregulated *CYP7A1* expression and blocked the effect of FGF-19 signalling in hepatocytes. Thus, they demonstrate a potential role for BA, adiponectin and FFA/NEFA in the pathogenesis of NASH, whilst also showing how increased FFA may disrupt the regulation of hepatic BA synthesis by FGF-19, although a full explanation of this mechanism is still not known. This study has not demonstrated any correlations between FGF-19, adiponectin, NEFA or M30 but future studies are required to test these relationships more robustly.

6.6.8 Limitations

One major criticism of this study is the lack of a control group of non-obese patients not undergoing any intervention. In the absence of an accepted and widely used reference range of normal values of the various biomarkers used in this study, use of a negative control group establishes a site-specific normal range

and is an additional form of validation. Unfortunately at the time of inception of these studies, no ethics approval or extra funding was sought for these additional assays. The biomarker values presented above do fall within the range of the published values and so these results do still have merit.

Although the main aim of this study was to evaluate changes at 6 months after surgery, it is pertinent to note that the metabolic effects of LSG evolve over a longer time period. In common with many other cohort studies in bariatric surgery, the 6 month interval studied here may be too short. Steinert *et al* noted that total BA concentration peaked at 1 year after both LSG and LRYGB **(458)**. Similarly, Illan-Gomez *et al* found that adiponectin levels were highest and IL-6 lowest at 12 months after bariatric surgery, with significant changes between 6 and 12 months **(99)**.

The changes in BA profile described here are novel and interesting. As well as performing studies with larger sample sizes over a longer time period, it will be important to measure dynamic post-prandial changes in BA levels, in conjunction with changes in gut hormone release. Steinert *et al* have shown that post-prandial incretin release increases within one week of LSG and is associated with a decrease in fasting insulin, before any changes to BA levels are seen **(458)**. This indicates that there may not be a direct relationship between BA metabolism, changes in gut hormones and improvement in insulin resistance. Furthermore, any exploration of potential hypothesis to explain the relationship between individual BA, changes in gut microbiota and improved markers of NAFLD/NASH will benefit from measurement of circulating LPS, as a marker of gut bacterial load, and 7alpha-hydroxy-4-cholesten-3-one (C4), which is an indirect measure of BA synthesis via the action of the rate-limiting enzyme *CYP7A1* **(448)**. Measurement of faecal BA levels will also improve the understanding of changes in BA absorption after LSG **(480)**.

6.7 Conclusions

This study confirms that LSG results in significant weight loss and improvement in glycaemic control after 6 months. These changes are accompanied by reduction in low-grade inflammation, as indicated by falling CRP, IL-6 and Leptin, but not markers of oxidative stress. CK-18 fragment levels also decrease, indicated a likely improvement in liver steatosis, although other supporting evidence for reduction in liver fibrosis, such as a fall in HA, was not seen. LSG was also noted to have an effect on BA metabolism and cycling, with a significant increase in FGF-19 indicative of increased bile flow and changes in the concentrations of four BA. The reduction in inflammatory markers correlated with fall in BMI but no association was seen with other markers or HOMA-IR. BA profile changes are associated with changes in cytokine levels and CK-18 M30 and these results should stimulate further research focussed on the effects of BA after LSG.

CHAPTER 7:

CONCLUSIONS

7.1 Overall conclusions from this thesis

The main aim of this thesis was to determine the extent and clinical significance of post-operative inflammatory response and metabolic changes in morbidly obese patients with fatty liver disease. As discussed at length in the introductory chapters, there are many studies investigating the changes in cytokines and other biomarkers after bariatric surgery, the aetiology and progression of NAFLD/NASH, and also of laparoscopy-induced oxidative stress and inflammatory response. The studies presented here are the first attempt to amalgamate these different issues and investigate their relationships in one cohort of patients. The patients recruited were in the "super"-obese category, with a mean BMI>60 kg/m^2 and this itself is novel as most similar studies include less obese patients.

The NAC Trial results showed that N-acetylcysteine did not reduce post-operative inflammatory response or liver transaminase rise, compared with controls. This conclusion can be drawn from the results of a comprehensive range of clinical, oxidative stress and cytokine markers. Nevertheless it is clear that there were limitations in both study design and study conduct. There was a lack of standardisation of the intraoperative insult and no stratification according to severity of NAFLD/NASH. These limitations may have been counterbalanced with a larger sample size and with recruitment of patients undergoing lengthier surgeries, such as LRYGB or laparoscopic BPD-DS.

The NAC Trial did reveal some interesting new observations about the post-operative response. Transaminase rises, corroborated by a rise in CK-18 fragments, were dramatic over the first day after surgery, indicating a significant liver injury is incurred during surgery. This injury may be a form of ischaemia-reperfusion injury, given the timing and pattern of M30 and M60 changes. The injury is associated with a rise in both pro-inflammatory cytokines, such as IL-6

and CRP but also a rise in IL-10 and adds to the growing body of evidence that cytokine changes cannot easily be divided into purely pro- and anti-inflammatory actions **(351)**. The interpretation of changes in oxidative stress markers was less straightforward and both the literature review and these results indicate that further work is required to identify and standardise which markers should be used. There was an excess of complications in the NAC trial and it would be interesting to definitively rule out any adverse effects of NAC. A trial of NAC in severe acute pancreatitis showed a higher mortality in the NAC group, although that study also closed before the planned sample size was reached {Siriwardena, 2007 #225}.

Most importantly, the biomarker changes did not appear to have any impact on the clinical outcome. Recovery to baseline occurred in most markers within 1-2 days of surgery. Given the good safety profile of bariatric surgery **(36)**, large scale comparative studies using clinical endpoints (such as rate of infective complications, length of stay or mortality) would require many hundreds of patients to demonstrate if a particular intervention is effective in improving outcomes. Biomarker outcome measures are poor surrogate endpoints in this context. However, intra-operative liver injury during laparoscopic bariatric surgery may have potential as an in vivo human model of steatotic liver IRI, to investigate the clinical efficacy of other potential therapeutic interventions that have proved effective in experimental animal models.

One further question remains about the importance of post-operative inflammatory response as a natural and necessary defence mechanism. At present these mechanisms are poorly understood and modulation of the immune response by targeting specific pro-inflammatory cytokines has had little clinical success {Hutchins, 2014 #634}. Abrogation of the inflammatory response may even have deleterious effects and this hypothesis deserves further study.

The longitudinal results confirmed the findings of other studies that bariatric surgery leads to significant improvements in glycaemic control and reduced inflammatory markers. The fall in CK-18 fragments suggests that there may be improvements in liver pathology beyond simply reduction in the degree of

steatosis, but there is a pressing need to corroborate these conclusions with other measures of liver fibrosis and inflammatory changes. There is little published work on bile acid profile after LSG and these findings are of great interest **(451, 458)**. This is the first study in patients undergoing LSG to present such a detailed analysis of individual BA changes. Although these findings, especially the correlations with other biomarkers, need to be confirmed in larger studies, they substantiate the concept of alterations in BA metabolism being important in the pathogenesis of fatty liver disease **(485)**. Taken together with the published trials using exogenous synthetic BA to treat NAFLD/NASH, these results should stimulate further interest in this field.

Overall, the studies presented in this thesis have shown that laparoscopic bariatric surgery has a significant post-operative inflammatory response and does cause liver injury, but that these changes are of a short duration. In the longer term, the overall health of patients improved dramatically, including resolution of co-morbidities and significant improvements in glycaemic control, markers of inflammation and liver injury.

7.2 Future work arising from this thesis

Building on the work in the NAC Trial and the longitudinal study presented in Chapter 6, there are a number of research questions that have arisen that merit attention:

1. Would a larger trial, possibly multi-centre, using a wider range of operations, including LRYGB, help to definitively answer if NAC has a role as a hepato-protectant during laparoscopic bariatric surgery?

The initiation of another NAC Trial focussing on extending the indications and recruiting a larger number of patients is under consideration. Measurement of intraoperative liver ischaemia and blood flow using newer devices, such as laser

Doppler fluximetry **(328)**, may help to both standardise the intraoperative insult and determine if NAC improves liver blood flow in this setting.

2. How do markers of liver injury, NAFLD and NASH change (deteriorate or improve) during the first few weeks after bariatric surgery, and is this related to changes in other biomarkers or the alterations in glycaemic control and/or BA metabolism?

This would involve another longitudinal cohort study with increased frequency of biomarker and BA measurement, at weekly or monthly intervals. The main benefit would be to identify the most vulnerable period after surgery for the liver. There is concern that some patients experience worsening of liver fibrosis following surgery **(218)**. This may be associated with specific events, such as infections, during the post-operative period that act as the "second hit" in the evolution of NASH/cirrhosis **(128)**.

3. How does LSG improve diabetes?

There are a number of studies investigating the rapid change in insulin resistance following RYGB that merit repeating in patients after LSG, including dynamic measurements of post-prandial insulin/glucose/incretin kinetics, changes in BA and alterations in lipolysis **(56, 465, 493)**. It is increasingly recognised that LSG may lead to weight loss through changes in gut hormones, increase in intestinal transit and unidentified neurohormonal mechanisms, and not just through mechanical restriction of intake. As discussed in Chapter 6, the design of BA studies can be improved by additional measurements of LPS as a marker of gut microbiota changes and C4, as a marker of BA synthesis **(494)**.

4. Potential role for non-hypothesis driven "-omic" studies.

The fall in NEFA demonstrated in this thesis indicates the potential role of changes in lipid profiles as a mechanism for other metabolic changes. Repeated lipidomic investigations in a cohort of bariatric patients may reveal that individual lipid species play a significant role **(495, 496)**.

There is also great interest in the role of microRNAs, small non-coding fragments of nucleic acids, which are involved in the regulation of expression and interaction of other genes. A number of microRNAs have been implicated in progression of chronic liver diseases and there is also interest in identifying individual microRNAs as prognostic markers and measures of liver injury, fibrosis and cardiovascular risk **(497)**. Identification of potential candidate markers using array technology on both serum and tissue, liver and/or adipose, can then be followed up with serial serum assays for each potential microRNA marker **(498)**.

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APPENDIX

APPENDIX 1:

PRESENTATIONS AND PUBLICATIONS ARISING FROM THIS THESIS

Parts of this thesis have been presented in poster form at the following meetings:

International Federation of Surgical Obesity, Hamburg 2011
Association of Upper Gastrointestinal Surgeons, Belfast 2011
American Association for the Study of Liver Diseases, San Francisco, USA 2011
British Obesity and Metabolic Surgery Society, Bristol 2012.

The results contained in this thesis have been published (in part) in abstract form:

Reduction in pro-inflammatory cytokines after weight loss surgery: a prospective study
Belgaumkar A, Carswell K, Mitry R, Hughes R, Dhawan A, Patel A
Gut 2012; 61:Suppl 2 A93

The effect of intraoperative N-acetylcysteine on hepatocellular injury during laparoscopic bariatric surgery - a randomised controlled trial
Belgaumkar AP, Carswell KA, Mitry RR, Hughes RD, Dhawan A, Patel AG
Gut 2011; 60:A1
Hepatology 2011; 54(S1): 690

The effect of laparoscopic sleeve gastrectomy on the entero-hepatic circulation of bile acids
Belgaumkar A, Vincent R, Carswell K, Mitry R, Dew T, Le Roux C, Patel AG
British Journal of Surgery 2011 98(S7): 3

Plasma Bile Acid profile is not altered by Laparoscopic Sleeve Gastrectomy
Belgaumkar A, Vincent R, Carswell K, Mitry R, Dew T, Le Roux C, Patel AG
Obesity Surgery 2011, 21: 1072

Bile acid absorption does not change after Laparoscopic Sleeve Gastrectomy
Belgaumkar A, Vincent R, Carswell KA, Mitry R, Dew T, Le Roux C, Patel A
Obesity Surgery 2011, 21: 1106

APPENDIX 2:

NAC TRIAL DOCUMENTATION

B1.1 Sponsor details

The formal details of the trial, hitherto referred to as the NAC Trial, are given below.

Sponsor:
King's College Hospital NHS Foundation Trust
Sponsor Contact – Jackie Pullen
King's Health Partners (KHP) Clinical Trials Office
F16, Tower Wing
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Email: jackie.pullen@kcl.ac.uk

EudraCT Number:	2008-001677-15
Trial Number:	ISRCTN81349394
Research Ethics Committee Reference:	08/H0808/2

Study Initiated: 07Jul2009 Early Termination Date: Aug 2012

B1.2 Investigators and Study Administrative Structure

Chief Investigator/Principal Investigator: Mr Ameet Patel Consultant Surgeon

Co-ordinating Investigator: Mr Ajay Belgaumkar, Clinical Research Fellow

Co-investigators: Ms Kirstin Carswell, Clinical Research Fellow

Laboratory co-investigators: Dr Ragai Mitry
Dr Robin Hughes
Professor Anil Dhawan

Trial Steering Committee Members: Mr Ameet Patel
Mr Ajay Belgaumkar
Ms Kirstin Carswell

KHP Clinical Trials Office Monitors Ms Hannah Mason
Ms Ingrid Brumarescu

KHP Clinical Trials Office Quality Manager Ms Jackie Pullen

B1.3 Definitions of Complications occurring within 30 days of surgery

Major

Anastomotic leak – objective evidence of leakage (either on contrast radiography or visual confirmation at re-operation) with or without clinical signs and symptoms.

Fluid collection - CT scan or ultrasound presence of fluid collection at least 5 cm in diameter with or without clinical relevance.

Hemorrhage - Requirement of at least 3 units of packed Red blood cells (1000 mL) within 24 h after the operation.

Chest Infection – signs of sepsis or septic shock accompanied by clinical signs and symptoms of chest infection

Sepsis – pulse >90bpm, temperature <36^{o}C or >38^{o}C, respiratory rate >20 or paO_2, White cell count <4 or >12000cells/mm^3 in presence of proven infection

Renal Failure – Creatinine > 150 or doubling of pre-operative value, persisting for at least 72 hours

Myocardial infarction – relevant ECG changes accompanied by clinical symptoms and signs, with a raised Troponin I

Pulmonary embolism – relevant clinical signs and symptoms with corresponding appearance on Computed Tomography (CTPA) or Ventilation/Perfusion scanning

Delayed gastric emptying: failure to resume oral liquid intake by postoperative day 10, and/or emesis over 500 ml on or after postoperative day 5, and/or continued nasogastric drainage >500 ml on or after postoperative day 5.

Cardiac Arrhythmias – ECG-proven new onset arrhythmias, eg. atrial fibrillation

Minor

Wound infection – erythema, purulent discharge and pain at wound site persisting for more than 48 hours

Post-op pyrexia – temperature above 38^{o}C lasting less than 48 hours with no progression to sepsis or accompanying clinical signs of local infection

Deep vein thrombosis – relevant clinical symptoms with sonographic evidence of thrombosis in the absence of signs of suspected pulmonary embolism

B1.4 Protocol Changes

The various protocol changes are described, including amendments that were required before and at the start of the NAC Trial for completeness. These were mainly minor changes.

B1.4.1 1st Substantive Protocol change:

Protocol amendment:	dated 1st April 2009
Old Protocol version:	Version Final 1.0 (01May08)
New Protocol version:	Version 2.0 (01Apr09)

1. Major change to inclusion/exclusion criteria.

Sleeve gastrectomy operation was included in the study as this had become an increasingly common operation performed on patients in the unit over the preceding year.

2. Minor clarification of one exclusion criterion.

The exclusion criterion of psychiatric illness was clarified to specify exclusion of patients with active illness. Many morbidly obese patients have a history of psychiatric illness, either as a causative factor or a sequel of obesity. It was obviously not practical to include patients with ongoing active psychiatric symptoms, as the validity of the assessment of their autonomy and ability to consent would have been in question.

3. Correction of inconsistencies between text and schedule of investigations in the protocol.

Various transcription errors between various parts of the protocol were corrected.

4. Change to AE reporting guidelines in the protocol.

It was stipulated that common surgical post-operative complications would be recorded as AEs but not necessarily SAE, unless they fulfilled the SAE criteria. Indeed, this part of the protocol probably should have been revised further as some post-operative issues, such as wound pain and nausea, are universal and classification of them as AEs was probably not appropriate.

5. Change to blinding procedure.

Text changes were as follows:
Version 1: The researcher will be blinded to the intervention until after data collection and sample analysis has occurred. This blinding will occur by marking the data and samples with a pre-arranged identification code, which will be contained within the envelope for randomisation.
After data collection and sample analysis has occurred, the research will be made aware of study allocation, so that comparative statistical analysis can be undertaken.

Version 2: After data collection and sample analysis has occurred, the researcher can check the randomisation schedule and will be aware of study allocation, so that comparative statistical analysis can be undertaken.

This is discussed above. Briefly, it became apparent that Pharmacy would require some time to dispense the IMP. Also, organising the appropriate per protocol dispensation of IMP would have to be the Research Fellow's responsibility due to the clinical commitments of all other parties, including Operating Surgeon and Anaesthetist.

6. Correction of typos/inconsistencies in protocol.

B1.4.2 2nd Substantive Protocol change

Protocol Change:	dated 26th July 2009
Old Protocol version:	Version 2.0 (01Apr09)
New Protocol version:	Version 3.0 (26Jul09)

1. Change to inclusion criteria (upper age limit)

The upper age limit was increased to 75 from 65 years in line with the evolving clinical practice of the Unit.

2. Change to unit of measurement of alcohol intake in exclusion criteria

This was changed to "units" as defined as being approximately 8-10g of alcohol, corresponding to 1 glass of wine, a 25ml measure of whisky or approximately half a pint of lager.

3. Extra study-specific blood test to be taken 96hrs post-surgery

It had always been the Investigators intention to take study-specific blood tests up to 4 days after surgery but this was left out in error during previous protocol versions.

4. Clarification of when participants are approached about the study

Text changes were as follows:
Version 2: Patients seen in the Multidisciplinary Morbid Obesity Clinic at King's College Hospital, who are being referred for morbid obesity surgery, will be eligible for inclusion
They will have until the time of their admission (usually 10-12 weeks) to make their decision. They will have a second opportunity to discuss the study further at the Pre-operative Assessment Clinic, approximately 1 week prior to admission.

Version 3: Patients seen in the Multidisciplinary Morbid Obesity Clinic or Pre-operative Assessment Clinic at King's College Hospital, who are being referred for morbid obesity surgery, will be eligible for inclusion.
They will have until the time of their admission to make their decision. There will be another opportunity to discuss the study at the Pre-operative Assessment Clinic.

This change was required as the timings between initial assessment and allocation of the date on the waiting list became more unpredictable due to organisational changes in practice, NHS targets and the ad hoc availability of extra operating lists. Nevertheless, the importance of giving potential participants multiple opportunities to discuss the study and adequate time to make decisions regarding their consent to participate was still pre-eminent.

5. Clarification about running time of 2nd NAC infusion

Version 2: Subjects randomised to the intervention will receive N-acetyl cysteine infusion, at standard 150mg/kg in 200ml 5% dextrose over 15mins at induction of anaesthesia followed by an infusion of 50mg/kg in 500mls of 5% dextrose during surgical retraction of liver for a maximum of 4 hours, together with standard anaesthetic medications.

Version 3: Subjects randomised to the intervention will receive N-acetyl cysteine infusion, at standard 150mg/kg in 200ml 5% dextrose over 15mins at induction of anaesthesia followed by an infusion of 50mg/kg in 500mls of 5% dextrose during surgical retraction of liver for a maximum of 4 hours, together with standard anaesthetic medications. *If surgery lasts for less than 4 hours, the infusion will be continued until it finishes.*

The last sentence was added as the length of operating became increasingly shorter over the preceding year or so (as a natural consequence of increasing operative experience). Therefore this stipulation was required so that patients received the same dose of IMP.

6. Clarification of when concomitant medication information will be collected

Having not been stipulated clearly before, the following was added: Information shall be collected regarding all concomitant medications (including over the counter vitamins and supplements) that participants are taking from the time of screening to the end of their participation in the trial (Month 6 post-operation visit).

7. Removal of PIS, ICF and GP letter from protocol appendix

This was done to bring the protocol in line with the standard template.

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Table C.1 Summary of Studies including measurement of Bile Acids after Bariatric Surgery (Continued over 3 pages)

Reference and institution	Year	Groups	Average BMI	Diabetics	Method of Bile Acid measurement	Timing of Measure	Dynamic Meal test?	Values	FGF19	Other markers measured	Significant Result summary
Ahmad Harvard/ MGH, USA	2013	5 RYGB 8 healthy controls	>35 vs 18-25 controls	Not defined	Reverse phase HPLC-MS	Preop, 1, 4 and 40 weeks postop	yes	taurineBA lower in obese vs lean control blunted rise in obese but increased after RYGB, significantly increased AUC by week 40 vs preop and no difference cf lean controls absolute value 1.1 pre vs 1.9 post	no	4 day food diary and 3day Bouchard activity chart	Normalises the blunted post-prandial response of BA and increases fasting levels
Patti, Obesity Harvard/ MGH	2009	>2yrs post RYGB n=9, 5 morbidly obese pts matched to Pre-op wt, 10 overweight pts matched to post-op weight	RYGB – post-op BMI 29.9, pre-op 50.2	No diabetes/ IGT	HPLC-MS	Cross-sectional design, fasting	no	Graphed, ≈8.5 RYGB vs ≈4 in obese and overweight groups	Yes, no sig diff betwee n groups	GLP1, Leptin, Adipnectin, TSH	totalBA higher in RYGB compared with weight matched controls inverse correlation with TSH, 2hour glucose and positively correlates with GLP-1 and adiponectin
Jansen, Digestive Diseases, Amsterdam Netherlands	2011	35 RYGB 61 healthy volunteers for control	47.6	Yes, not specified, HOMA-IR 7.3 preop	Enzymatic cycling method	Pre-op 3months	No	BA sig increase 4.5 to 8.5	YES, 0.16 to 0.23	FGF21 higher postRYGB, Hepatic FGF 21 mRNA higher in obese vs 10 liver resection controls, MRI TESLA shows fall in hepatic fat 14.4% to 5.8%	TotalBA and FGF 19 & 21 higher after 3months and associated with fall in HOMA-IR and hepatic fat content

Table C.1 Summary of Studies including measurement of Bile Acids after Bariatric Surgery (Continued)

Reference and institution	Year	Groups	Average BMI	Diabetics	Method of Bile Acid measurement	Timing of Measure	Dynamic Meal test?	Values	FGF19	Other markers measured	Significant Result summary
Gerhard *et al*, Diabetes Care, Danville, USA **(450)**	2013	186 RYGB (115 T2DM) 110 controls (66 T2DM)	48 vs 30	Yes forming 3 groups: NDM, DM with remission, DM no remission	LC-MS	Median 140-150 days post surgery	No	In T2DM, higher BA and lower FGF19 Lower FGF19 correlates with higher hepatic *CYP7A1* expression FGF19 and total BA increased after RYGB, larger increase in DMremission	yes	Hepatic expression *CYP7A1* and FXR, FGF4R etc	FGF19 and BA are lower in diabetics and increase more post RYGB in DMremission
Simonen *et al*, Obesity Surgery Kuopia, Finland **(457)**	2012	30 RYGB	46	Yes – mean HOMA-IR 6.58	Enzymatic for TotalBA HPLC-MS for fractions	Pre-op 1 year	no	Graphed 3.8μmol to 7, p=0.002 Individual BAs given, but no sig increase	no	Bioelectrical impedance (lean body mass) Indirect calorimetry with lipid and glucose oxidation Liver and Adipose RNA/PCR for	Rise in fasting total BA after RYGB Decreased levels of Taurine-BA associated with increased DIO2 expression in adipose tissue DIO2 correlates positively with lipid oxidation and negatively with glucose oxidation, altered substrate oxidation via changes in TGR5-DIO2 signalling
Pournaras *et al*, Endocrinology Taunton, UK **(456)**	2012	12 RYGB 6 LAGB non-obese controls	RYGB 49.8 LAGB 45.4	Yes but n not stated	LC-MS	Pre-op, day 4, day 42	no	Graphed ≈2, increasing to ≈5 after RYGB	Yes – lower than controls No changes in LAGB Sig increase in RYGB after 4 and 42 days	Dog and Rodent models of bile diversion	Increased FGF-19 and totalBA after RYGB at d4 and d42 In dog, combination of bile and food doubled GLP-1 and PYY response In rat, bile in ileum associated with increased GLP-1 and PYY and reduced food intake and body weight

Table C.1 Summary of Studies including measurement of Bile Acids after Bariatric Surgery (Continued)

Reference and institution	Year	Groups	AverageBMI	Diabetics	Method of Bile Acid measurement	Timing of Measure	Dynamic Meal test?	Values	FGF19	Other markers measured	Significant Result summary
Nakatani *et al*, Metabolism Tokyo, Japan **(454)**	2009	34pts 19 "malabsorptive" RYGB and BPD 15 "restrictive" LSG and LAGB	43 vs 45	16/19 IGT-DM 10/15 IGT-DM	HPLC	Preop, 1 and 3months post op	No	BA sig increased after MP and RP 3.1,7.5, 9 vs 3.2, 9.4, 12.6	no	Adiponectin, leptin, GLP-1, GIP, Lipids	BA correlate with GP, GLP-1, Adiponectin
Kohli *et al*, Endocrinology Cincinnati, USA **(499)**	2013	18pts, 8RYGB, 10 LAGB	Not given	Excluded	Electrospray ionisation LC-MS	Preop and after 20% weight loss	Yes at 1 hour after mixed meal	Graphed Fell non-sig after LAGB from 1.8 to 0.9, increased significantly 0.9 to 2.1 after RYGB, similar post-prandial rise significantly higher	No	GLP-1, TSH, Kir6.2 and COX IV skeletal muscle increased after RYGB, not LAGB (TGR5 downstream elements) Resting energy expenditure decreased post-op	BA and TGR5 signallling increased after RYGB BA correlate with GLP-1 but unrelated to fall in REE
Steinert *et al*, Obesity, Basel, Switzerland **(458)**	2013	RCT – n=7 RYGB vs n=7 LSG 14pts 6 healthy controls	RYGB – 50 LSG 43	Yes – mean pre-op HOMA=9 n per group not specified	Single-ion GC-MS PLASMA	Pre, 1week, 3months and 12 months	0, 15 and 180mins	Healthy >Obese (graphed – fasting total BARYGB ≈0.8 to ≈ 2; LSG ≈0.8 to ≈1.8) TotalBA higher in RYGB only at 1yr LSG no sig difference	No	GLP-1, PYY No sig correlation with BA Total BA negative correlation with BMI	BA not increased after LSG Not correlated with diabetes or GLP-1,PYY
Haluzikova *et al*, Obesity, Prague, CZ **(451)**	2013	17 female LSG 15 healthy controls	LSG 43.2 vs 22 controls	No	GC/MS	Pre-op, 6, 12 (&24) months	No	Lower than controls, no sig change total BA Graphed ≈2-3	Yes Lower than controls, sig increase after 6month	Adipokines, ghrelin, CRP, subcut fat adipokine mRNA	Increased FGF19,21, Adiponectin, reduced ghrelin, leptin No change in BA